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A TEST OF THE INDUCTIVE EFFECT OF PENTOSE NUCLEIC ACID OF THE KIDNEY ON THE ISOLATED ECTODERM[1]

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INTRODUCTION

TOIVONEN (1940) implanted a piece of the guinea pig kidney in the blastocoel of *Triturus*-gastrula, and obtained frequent induction of somites, spinal cord, deuterencephalon and ear vesicle. The same effect was observed, when it was tested on the isolated ectoderm (YAMADA 1952, YAMADA and TAKATA, in press). Analysing the factors responsible for the induction, the present authors found that fractions rich in pentose nucleoprotein possessed this inductive ability (loc. cit.). The fact raised the question: Is the pentose nucleic acid (PNA), contained in this tissue, responsible for the induction? Two lines of attack were tried: In one group of experiments, PNA separated from those fractions was tested on the isolated ectoderm for its inductive ability. In the other group of experiments, PNA was removed from those fractions by treating them with crystalline ribonuclease, and then the effect of thus treated fractions was tested on the isolated ectoderm. Further experiments with some proteolytic enzymes provided us with relevant data for the discussion of the problem. In the present paper, the first group of experiments alone will be reported.

TECHNIQUE

To test the inducing ability of PNA, a piece of solid sample was put directly on the internal surface of a piece of isolated ectoderm, and covered by another piece of ectoderm. The margin of two ectodermal pieces readily fused together to enclose the sample in an ectodermal jacket.[2] In this way the un-coated, internal surface of the ectoderm was exposed directly to the sample. The ectoderm was isolated from the earliest stage of the gastrula of *Triturus pyrrhogaster*

[1] Supported by a grant of the Rockefeller Foundation and the Research Expenditures of the Ministry of Education.

[2] This technique was preferred to that of testing the sample in solution added to the culture medium, because, immediately after the isolation, the ectoderm curls up into a sphere, and is covered perfectly with the surface coat, which is in all likelihood impermeable to nucleic acid in the medium. However one technical possibility for testing the sample in solution on the ectodermal cells, seems to be opened by the method worked out by NIU and TWITTY (1953). We are trying to adapt this method to our particular problem (see later).

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(*cf.* YAMADA 1950 *a* and *b*). The same technique was used, when we demonstrated the inductive effect of kidney tissue and its fractions on the isolated ectoderm (YAMADA 1950 *b*, 1952, YAMADA and TAKATA, in press). However, as shortcomings of such technique the following points must be born in mind: (1) It is very difficult to determine the effective quantity of testing substance; (2) The delicate internal surface of ectoderm might be damaged by the direct contact with the solid sample, and be lead to the cytolysis. As the sub-cytolytic stimulus can lead to a neural differentiation with archencephalic features (HOLTFRETER 1944, 1945, YAMADA 1950 *a*) results of experiment must be studied critically.

For the details of operation technique readers are referred to the earlier papers of some of the writers (YAMADA 1950 *b*, YAMADA and TAKATA, in press). As the operation and culture medium HOLTFRETER solution was used which was buffered with sodium bicarbonate to pH 7.2–7.3. Exceptionally in the experimental series I*b* this standard pH value was not used.

Highly polymerized PNA was prepared by the method of GRINNAN and MOSHER (1951). The kidney of adult guinea pig weighing about 200 g. was used as the source. Kidneys were removed from freshly decapitated exsanguinated animals, and washed repeatedly with ice-chilled saline solution. 40 g. of tissue was homogenized for 30 seconds with 2.5 volumes of cold 0.4 M NaCl in a waring blender which had been kept at −20° C. After 30 min. stirring at 0° C., the homogenate was centrifuged at 0° C. at 3,000 r.p.m. for 25 min., and the residue was again extracted with the same volume of 0.14 M NaCl. The combined extract was centrifuged as above, small amount of sediment being discarded. Solid guanidine hydrochloride was added to the extract so that the final concentration of the extract was 4 M with respect to guanidine hydrochloride. After neutralization to pH 6.8 (glass electrode) with dilute NaOH, the mixture was incubated at 38°-40° C. for 1 hour. It was then chilled, and centrifuged at 3,000 r.p.m. for 10 min. and the supernatant was discarded. The residue, thus obtained, was washed once with 4 M guanidine hydrochloride and twice with 0.14 M NaCl. This was then suspended in 20 cc. of 0.14 M NaCl[3] and was subjected to the repeated deproteinization with chloroform-butanol according to SEVAG. Sodium salt of PNA wsa precipitated from the aqueous phase by adding 3 volumes of 95% ethanol. The PNA was redissolved in cold distilled water, and the small amounts of impurity were centrifuged down. PNA was reprecipitated with a few drops of 1 M NaCl and 2 volumes of 95% ethanol. It was then washed twice with absolute ethanol and once with ether, and dried *in vacuo*. This sample was tested in the experimental series I.

The PNA sample, thus prepared, revealed a positive pentose reaction with orcinol test, characteristic ultraviolet spectra of nucleic acids, and a negative reaction both to DISCHE's test of desoxypentose and biuret test of proteins.

Calcium salt of PNA was precipitated from the aqueous solution of thus prepared PNA with 2 volumes of 95% ethanol in the presence of $CaCl_2$. This sample was used as implants in the series IV.

In other series of operation, samples of PNA separated from the same tissue according to KERR and SERAIDARIAN (1949) was used. The scheme of the procedure was as follows: The kidney of guinea pig taken out immediately after killing was chilled with ice, freed from connective tissues and fat, minced thoroughly and weighed. After addition of 1.5 volumes of

[3] The heating at this step, which is necessary for a good yield, was dropped for the sake of avoiding possible change in specific morphogenetic effect of PNA prepared.

cold 0.14 M NaCl solution to the minced tissue, the mixture was stirred mechanically for one hour in a refrigerator and then centrifuged at 3,000 r.p.m. for 30 min. at 1° C. The residue was re-extracted in the same way. An aliquot of the combined extracts was brought to pH 4.2 (glass electrode) by addition of N HCl. The acid precipitate was centrifuged down at 4,000 r.p.m. for 30 min. at 1° C. and the supernatant was discarded. After dissolving the obtained precipitate in small volume of cold 0.14 M NaCl solution, N NaOH was added to adjust the solution to pH 6.8 and NaCl was added to make 3 M. The mixture was then shaken during 36–40 hours at 6° C. in a refrigerator to permit the dissociation of PNA from protein. 0.1 N HCl was added to the obtained filtrate to adjust it to pH 4.2. After removal of precipitate by centrifugation the sodium nucleate in the supernatant was precipitated by adding 3 volumes of cold 95% ethanol, and then separated by centrifugation. The sample was purified by repeating the acid-precipitation and ethanol-precipitation, and tested in the experimental series II. An aliquot of this sample was used to prepare lanthanum salt, which was precipitated from a suspension of PNA in 0.14 M NaCl by adding 2% La-acetate solution. This La-salt sample was used as implants for experimental series III after washing in 0.14 M NaCl solution and 95% ethanol and drying *in vacuo*.

The implant was prepared by cutting dried PNA under a binocular microscope with a fine dissecting lancet. The approximate size of the implant was 0.25–0.30 mm^3. A spectrophotometrical estimation of some samples of implants, thus prepared, gave the following data: PNA-P/1 implant = 0.307 ± 0.060 μg.

The pieces of isolated ectoderm, to which this amount of PNA was implanted, had the approximate diameter of 0.8–1.0 mm.

Experiments

A. Control Series

The presumptive ectoderm of the earliest gastrula of *Triturus pyrrhogaster* was isolated, before coming in contact with the invaginating endo-mesoderm and cultured in Holtfreter solution, which was adjusted with sodium bicarbonate to pH 7.2–7.3. All of 128 explants, thus cultured at room temperature for 7 to 14 days, differentiated into aggregates of epidermis cells with characteristically wrinkled surface. Some cell debris or large spherical free cells were encountered within vesicles or meshwork of epidermis. No neuralized cells could be recognized in the explants.

B. The Effect of Na-Salt of PNA of the Kidney Prepared with Guanidine Hydrochloride-Technique (Series I *a*, I *b*)

The dried sample of PNA prepared from the kidney of guinea pig according to the method of Grinnan and Mosher was cut in minute pieces, put in 70% ethanol for several minutes, washed thoroughly in sterile Holtfreter solution and then implanted as a whitish half-transparent gel in the isolated ectoderm. Within three days the explants developed more or less conspicuous wrinkles on their surface. The culture extended from 8 to 11 days at 18° C., except for two explants which must be fixed on the 7th day. As shown in the table (Series I *a*), the frequency of induction was extremely low. A small brain-type induction, accompanied by a nose-type, in one explant was the only case of definite induction. Another explant contained a cell-condensation without any neural

character. All other explants contained nothing but epidermis cells, which often formed a conspicuous vesicles.

Table 1. Effect of implantation of PNA on the differentiation of the explants of the gastrula ectoderm, compared with the effect of the original tissue and its extract

Series	Nature of implants	Number of explants					Remarks
		Available explants	With any induction	With archencephalic induction	With deuterencephalic induction	With spinocaudal induction	
Control	—	128	0	0	0	0	
I *a*	PNA, Na-salt	17	1	1	0	0	Standard condition
I *b*	PNA, Na-salt	18	11	9	0	0	Cultured at pH 7.7
II	PNA, Na-salt	8	0	0	0	0	Standard condition
III	PNA, La-salt	43	3	3	0	0	Standard condition
IV	PNA, Ca-salt	30	0	0	0	0	Standard condition
Ne	Kidney tissue	32	31	0	8	29	Data from YAMADA and TAKATA, in press.
A	0.14 M NaCl extract	71	65	0	23	59	Data from YAMADA and TAKATA, in press.

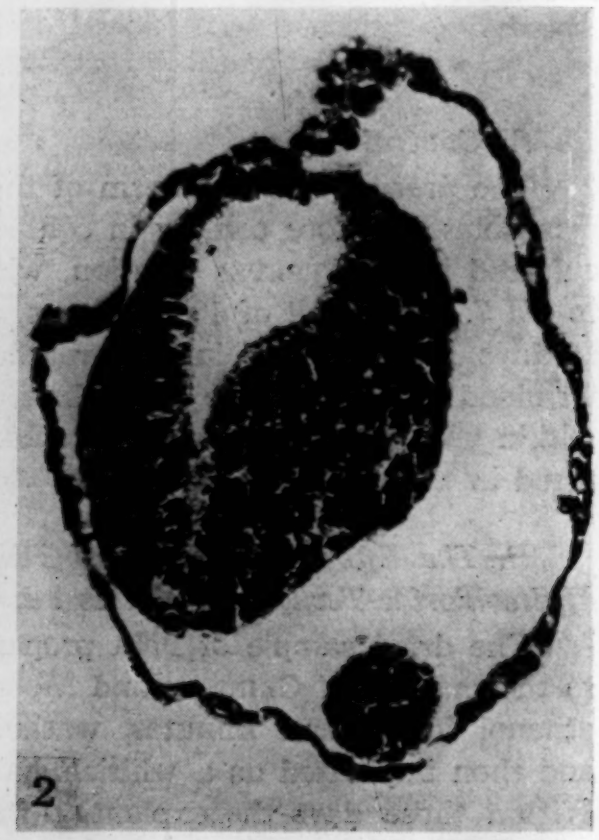

Fig. 1 (*left*). A rather irregular brain-like structure, not covered by the epidermis, differentiated from the isolated ectoderm implanted with Na-salt of PNA (series I *b*). ×150.

Fig. 2 (*right*). A rather well-formed archencephalon-type structure, accompanied by a small nose-type structure, differentiated from the isolated ectoderm implanted with Na-salt of PNA (series I *b*). ×150.

In above experiments operation and culture were carried out in a HOLTFRETER solution adjusted to our standard pH value, *i.e.* 7.2. Implantation experiments of the same sample carried out at pH 7.7 gave however a quite different results (Series I *b*). Of 18 available explants 10 showed clear neural induction, 5 could be classified into the archencephalic type including archencephalon-type (Fig. 2), eye-type and nose-type (Fig. 2), while 5 other cases represented irregularly structured neural mass (Fig. 1), often characterized by a large number of small cavities. Another explant showed cell-condensation without clear neural differentiation. The rest of the explants, seven in number, differentiated wholly into epidermis.

These results might suggest a significance of pH of the medium for the outcome of induction experiments of this sort. However the number of experiments is too small to allow any definite conclusion.

C. The Effect of Na-Salt of PNA of the Kidney Prepared after KERR and SERAIDARIAN (Series II)

The sample prepared as above indicated was desinfected in 70% ethanol, dried *in vacuo*, and was put directly on the internal surface of isolated presumptive ectoderm. The ectodermal jacket, thus produced, was cultured at room temperature for 8 days. The explant differentiated epidermis with wrinkled surface, which could not be distinguished in any way from that of the control series. No trace of neural tissue could be identified in the explants.

D. The Effect of Lanthanum-Salt of PNA Prepared after KERR and SERAIDARIAN (Series III)

Sample of lanthanum salt of PNA prepared as above described was tested. In all, 43 explants were cultured for 9 to 10 days at 20°–23° C. While the majority of explants differentiated only epidermis, three explants contained neural structures. The arrangement of the neuralized cells was irregular due to the presence of many minute cavities around which cells were scattered. Two structures were accompanied by the pigment layer (Fig. 3). The transparent cytoplasm and elongated nuclei in these structures suggested also the optic nature of this neural induction. In one of them a small lens-type structure was recognized. In 15 explants the implant was recovered in sections as basophilic mass embedded in the ectoderm. However, of three explants carrying induced structures, two were free from such implants.

E. The Effect of Ca-Salt of PNA Prepared by the Guanidine Hydrochloride-Technique (Series IV)

Ca-salt of PNA prepared as above was tested. 30 explants could be cultured for 8 to 10 days at 18° C and studied in sections. All explants differentiated atypic epidermis, with more or less pronounced wrinkled surface (Fig. 4). Many of them showed degenerating cells or cell-debris within the vesicles developed in the epidermis.

Fig. 3 (*left*). An irregular eye-type structure differentiated from the isolated ectoderm implanted with La-salt of PNA (series III). Note the "pigment layers," as black, irregularly elongated bodies. ×150.

Fig. 4 (*right*). An aggregation of epidermis cells, with a vesicle containing degenerating cells, differentiated from the isolated ectoderm implanted with Ca-salt of PNA. ×80.

Discussion

J. Brachet emphasized the role of PNA in the embryonic induction on the ground of various observations and experiments. Here we need to cite only his induction experiments (1942, 1945), which were believed to demonstrate the participation of PNA in the neural induction by the living organizer as well as by the heterogeneous inductors: (1) The devitalized organizer lost most of its inducing power after a treatment with ribonuclease; (2) Tobacco mosaic virus, PNA-containing granules of liver, kidney, gastrulae, etc. induced frequently neural structures when implanted in the young gastrulae. The liver granules and tobacco mosaic virus lost most of their induction power after a treatment with ribonuclease. Later experiments of Kuusi (1953), however, did not give results, which would be expected from the original idea of J. Brachet. Thus tobacco mosaic virus, which showed a higher frequency of archencephalic induction did not lose this power, even after the removal of PNA by extracting with 10% perchloric acid for 20 hours. She further found that the implantation of commercial PNA in albumin was followed by archencephalic induction in a high frequency. Yet the here used albumin alone caused archencephalic inductions in the control series. She thus concluded: "la presence du RNA peut augmenter la tendence archencéphalique, mais elle n'est pas obligatoirement nécessaire à la formation

des inductions archencéphaliques." She further cultured 16 explants in a solution of commercial PNA (0.5 mg/1 l.) but could not find significant difference in the frequency of neural differentiation toward the control. PNA extracted from the rabbit liver caused cytolysis but no neuralization in 6 explants of *Triturus helveticus* in a similar experiment. With explants from *Amblystoma mexicanum* gastrulae, cultured in a solution of the same PNA, she obtained some neuralization at a concentration of 0.2 mg/1 l. It should be noted, however, that the difference of the results toward control is hardly significant in view of small number of cases ($n = 6$). Some of those experiments are also included in a paper by J. BRACHET, T. KUUSI and S. GOTHIÉ (1952). Here they suggested that the disappearance of induction power of tobacco mosaic virus and tissue granules after treatment with ribonuclease, observed earlier by J. BRACHET, was perhaps due to a contamination of the ribonuclease sample used with proteolytic activity.

In the present experiment the isolated ectoderm must have been exposed to a high concentration of PNA[4] with its internal surface. However, it failed to show any morphogenetic reaction in most of cases. It must be remembered here that the PNA had been prepared from the tissue, which consistently showed a high frequency of spino-caudal and deuterencephalic induction under the same experimental condition (Table 1), and that the samples prepared with two different methods were tested in three different salts.

The neural induction obtained in series I *b* and III seems to be a dorsalization due to sub-cytolytic stimulus exerted by the implant. Although series I *b* seems to suggest the importance of pH level of the medium for such dorsalization, we want to preserve a definite conclusion for further investigations. It might be pointed out, in this connection, that a high pH value tends to increase the probability of dorsalization by the sub-cytolytic stimulus. That such dorsalization of the ectoderm leads to archencephalic structures conforms very well to the earlier observations (HOLTFRETER 1944, 1947, YAMADA 1950 *a*) and to the theoretical standpoint expressed by the senior writer (YAMADA 1950 *a* and *b*). KUUSI also observed archencephalic structures in her previously cited experiments with PNA.

Taken altogether, our results do not seem to support the idea that PNA as such is the cause of the induction obtained by the kidney tissue. However, before a definite conclusion can be drawn, we have to consider the following possibilities: (1) PNA is actually active in bringing about the dorsal differentiation, obtained in the induction experiments with the tissue or extract. However, its action is in some way dependent upon the presence of proteinic factor in such a way that PNA alone cannot cause the induction, as shown in the present experiment; (2) The sub-cytolytic stimulus due to PNA is indeed responsible for the occurrence of induction in experiments with tissue and extracts. However, the presence of a proteinic factor in these inductors influences the outcome of induction in such a way that cytolytic effect of PNA is minimized and spino-caudal and deuterencephalic differentiation instead of archencephalic differentiation is initiated.

[4] *ca.* 0.3 μg PNA-P per explant (*cf.* p. 125).

Although these are interesting possibilities, the following results of experiments obtained at our laboratory seem to exclude them completely: The kidney tissue and its different fractions, with spino-caudal or archencephalic effect, lost their inductive effect after a extremely short treatment with crystalline trypsin, and also after a short treatment with crystalline chymotrypsin. Further, some fractions from the kidney with spino-caudal effects and one fraction from the liver with archencephalic effect retained their original strong regional inductive ability, after an almost complete removal of nucleic acids and nucleotides by a treatment with crystalline ribonuclease and desoxyribonuclease (HAYASHI, unpublished).

A series of induction experiments with turnip-yellow virus made by one of the present writers (YAMADA, unpublished) seem to complement the present results and idea. This virus has an extremely high content of PNA, which can be separated from the protein very easily without appreciable depolymerization. If the virus is implanted in the isolated ectoderm no induction followed. PNA separated from the virus also induced nothing in most of cases. However, when it was given in a condition, which caused an immediate partial disaggregation of the ectoderm, the explant later differentiated conspicuous archencephalic structures. Comparing these results with the present ones, it might be said that, regardless whether a nucleoprotein complex is inductive (kidney pentose nucleoprotein) or not (turnip-yellow virus), the PNA separated from it can cause neural differentiation under some special condition, perhaps through sub-cytolytic stimulus. It might be added, however, that we are still not absolutely sure that a perfectly pure pentose nucleoprotein from the kidney is inductive under our experimental condition, although all results, till now obtained, do suggest this possibility.

Before concluding, a recent work of NIU and TWITTY (1953) might be mentioned: If a small piece of gastrula ectoderm of *Triturus* or *Amblystoma* is cultured under such a condition that the individual cells are spread on the glass surface, the inductive effect of the organizer is mediated by a factor or factors which are diffusible in the culture medium. It was further reported that the medium "conditioned" by the organizer contains nucleic acid or related substance (NIU 1953). We are now running experiments, which should decide whether our PNA sample added to the culture medium can cause induction in the ectodermal cells under a similar condition. Obviously, the results of such experiments and our present ones need not be the same, as the experimental conditions are quite different in two cases.

SUMMARY

(1) Pentose nucleic acid was separated form the kidney of guinea pig by two different methods, and was tested for its inductive effect on the isolated presumptive ectoderm of the early gastrula of *Triturus pyrrhogaster*. The main aim of the experiment was to decide, whether the strong inductive effect of this tissue and its fractions containing pentose nucleoprotein is dependent on the pentose nucleic acid.

(2) Pentose nucleic acid implanted in the form of sodium-salt had an almost

negligible effect on the isolated ectoderm in two different experimental series (1 *a*, II). In the series, where the culture medium was adjusted to higher pH (7.7) than the standard (7.2), the same nucleic acid sample induced in an appreciable frequency archencephalic or irregular neural structures in the isolated ectoderm (series I *b*).

(3) Lanthanum salt of pentose nucleic acid showed archencephalic induction in a very low frequency and no other inductive effect, when tested on the isolated ectoderm (series III).

(4) Pentose nucleic acid implanted as Ca-salt did not show any inductive effect on the isolated ectoderm (series IV).

(5) A disccusion of the results obtained here, together with those of other related experiments, seems to indicate that pentose nucleic acid is not the active inductive component of the kidney tissue of guinea pig and its extracts under our experimental conditions.

Literature Cited

BRACHET, J., T. KUUSI and S. GOTHIÉ, 1952. Une étude comparative du pouvoir en implantation et en micro-injection des acids nucléique et des constituants cellulaires nucléoprotéiques. Arch. Biol., *63*, 429-440. — GRINNAN, E. L. and W. A. MOSHER, 1951. Highly polymerized ribonucleic acid: Preparation from liver and depolymerization. J. Biol. Chem., *119*, 719-726. — HOLTFRETER, J., 1944. Neural differentiation of ectoderm through exposure to saline solution. J. Exp. Zool., *96*, 251-340. — HOLTFRETER, J., 1945. Neuralization and epidermization of gastrula ectoderm. J. Exp. Zool., *98*, 161-209. — HOLTFRETER, J., 1947. Neural induction in explants which have passed through a sublethal cytolysis. J. Exp. Zool., *106*, 197-222. — KERR, S. E. and K. SERAIDARIAN, 1949. Studies on ribonucleic acid I. Preparation from pancreas. J. Biol. Chem., *180*, 1203-1208. — KUUSI, T., 1951. Über die chemische Natur der Induktionsstoffe. Mit besonderer Berücksichtigung der Rolle der Proteine und der Nukleinsäuren. Annal. Zool. Soc. Zool. Botan. Fennicae "Vanamo," *14*, 1-98. — KUUSI, T., 1953. Sur les effets des acides nucléiques et des protéines dans l'induction hétérogène. Arch. Biol., *64*, 189-226. — NIU, M. C., 1953. *In vitro* study of induction (Abstract). Anat. Rec., *117*, 560. — NIU, M. C. and V. C. TWITTY, 1953. The differentiation of gastrula ectoderm in medium condition by axial mesoderm. Proc. Nat. Acad. Sci., *39*, 985-988. — TOIVONEN, S., 1940. Über die Leistungsspezifität der abnormen Induktoren im Implantatversuche bei Triton. Ann. Acad. Sc. Fenn. Ser. A, *55*, 1-150. — YAMADA, T., 1950. Dorsalization of the ventral marginal zone of the *Triturus* gastrula, I. Biol. Bull., *98*, 98-121. — YAMADA, T., 1950. Regional differentiation of the isolated ectoderm of the *Triturus* gastrula induced through a protein extract. Embryologia, *1*, 1-20. — YAMADA, T., 1952. Nucleoprotein and regional induction (Abstract, in Japan.). Zool. Mag. (Tokyo), *61*, 115. — YAMADA, T. and K. TAKATA. An analysis of spino-caudal induction by the guinea pig kidney in the isolated ectoderm of the *Triturus*-gastrula. (in press).

THE MANIFESTATION OF THE URINOGENITAL PAPILLAE OF THE MEDAKA (*ORYZIAS LATIPES*) BY SEX-HORMONES

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Introduction

Extensive experimental analyses of the manifestation of external sexual characters of fish have been made especially of viviparous cyprinodonts. Some researches have been made on oviparous cyprinodonts. All of these were concerned with male-positive sex characters, such as the gonopodium or the anal fin of the male. The fact that these male-positive characters are manifested by male hormone has been conclusively proved through a number of investigators. Witschi and Crown (1937), Regnier (1938), Baldwin and Goldin (1940) in the swordtail (*Xiphophorus helleri*), Regnier (1938), Eversole (1939) and Hopper (1949 *a, b*) in the guppy (*Lebistes reticulatus*), Grobstein (1940, 1942) in the platyfish (*Platypoecilus maculatus*), Turner (1941), Okada and Yamashita (1944 *b*) in the top-minnow (*Gambusia affinis*), all published detailed studies on the masculinizing effect of testosterone upon the anal fin of females of viviparous cyprinodonts. Hildemann (1954) in *Lebistes reticulatus* showed that the caudal fin-ray prolongation, which is a male-positive sex character, is induced in methyl testosterone-fed adult females. Nagata (1934, 1936) and Okada and Yamashita (1944 *a*) published studies on masculine sex-characters of the oviparous cyprinodont (*Oryzias latipes*). We have excellent review by Okada (1943) in this field. Experiments reported herein are concerned with a female-positive sex-character of the medaka (*Oryzias latipes*).

Although the external sexual characters of this fish have already been described by Oka (1931, 1938), they will be given briefly here. The dorsal and anal fins of the male are longer than those of the female and at their distal margins have a saw-toothed appearance. The saw-toothed distal edges are due to pronounced elongation of rays of these fins in the male, and the dorsal fin of the male has a deep cleft between the fifth and sixth rays. The rays of the anal fin of the male, which are composed of more segments than those of the female, are usually single except in the last ray which is usually bifurcated. Except the first two or three rays these of the anal fin of the fully grown female are usually bifurcated in the distal region. On the rays of the posterior half of the anal fin, the male possesses numerous numbers of small papillar processes (designated as the PP). The process-bearing rays are usually extended from the second to the seventh or nineth ray counting from the posterior end of the

anal fin. Some full-grown males have the papillar processes also on the last ray. Guanophores which are pronouncedly distributed along the distal border of anal, caudal and dorsal fins of the male during the breeding season are not apparent or a few in the female. In the female the pelvic fins are more elongated than in the male.

OKA (1931) also observed the presence of a pair of nipple-like skin folds posterior to the anus in the female covering the genital pore. These urinogenital papillae (briefly designated as the UGP) are very pronounced structures in the female while the corresponding structure in the male is poorly developed.

NAGATA (1934, 1946) performed gonadectomy in the medaka and transplanted the testis into ovariotomized fish. According to his study, the manifestation and the maintenance of the papillar processes (the PP) in the male anal-fin are dependent upon the presence of the testis. He was able to induce the papillar processes in the female anal-fin by transplanting the testis into ovariotomized fish. OKADA and YAMASHITA (1944 *a*) repeated this experiment with the same result and extended experimental studies on the male-positive sex-characters. They showed that the subcutaneous administration of the synthetic androgen (Methyl-dihydro-testosterone) to the female caused the masculine shaped fins and produced the papillar processes in the anal fin. Their experiments are concerned with such male-positive sex-characters as larger anal and dorsal fins and with the presence of the papillar processes in the male.

No experimental study on the urinogenital papillae (UGP) of the medaka, which are female-positive sex-characters, has heretofore been made. There are three possible possibilities to account for the manifestation of the female-positive sex-characters: (1) they may be manifested in the female by the absence of the male hormone and their development in the male may be inhibited by the presence of the male hormone; (2) they may be manifested in the female by the female hormone and their development in the male may be inhibited by the absence of the female hormone, and (3) they may be manifested by both female and male hormones but their development is more sensitive to the female hormone than to the male hormone. The following experiments were undertaken to determine which possibility is plausible with respect to the UGP.

MATERIALS AND METHODS

Materials were the orange-red variety of the medaka (*Oryzias latipes*) and relatively large full-grown fish (30–45 mm.) were used in the experiments. The methods of gonadectomy and transplantation of gonad were essentially similar to those of NAGATA (1934) and OKADA and YAMASHITA (1944 *a*) with the exception that the operations were performed in Ringer's solution instead of in tap water. The fish were anesthetized with 0.035 per cent chloretone and were then imprisoned by pins in a small paraffine-wax dish contaning M/7.5 Ringer's solution. Ringer's solution has following composition: M/7.5 NaCl 100 parts + M/7.5 KCl 2.0 parts + M/11 $CaCl_2$ 2.1 parts.

In the experiment of gonadectomy, an incision is made into one side of the body extending from the base of the pectoral fin obliquely ventrad to the vicinity of the anus. The gonad is then drawn through the opening by forceps. In

transplanting the gonad, the excised ovary is cut into two pieces while in Ringer's solution and one-half of the ovary is implanted into the castrated male. By tying a silk thread around the abdominal region the wound is held closed. After completion of the operation, the fish is kept in Ringer's solution for two or three days. These fish are then reared in spring-water aquaria. In a successful operation, the wound heals within a few days and then thread is removed from around the body.

In administration of hormones, a pure powder of estrone or methyl testosterone was inserted subcutaneously between the scales. The length and thickness of the urinogenital papillae were measured with a micrometer under a low-power microscope. In order to study the structure of the UGP as well as reproductive organs, serial sections were made. The body length of fish described in the present is the measurement of the living fish from the snout to the distal edge of the caudal fin.

Urinogenital Papillae (UGP) of the Medaka

Robinson and Rugh (1934) in their study of the reproductive process of this fish remarked that the urinogenital papillae in the female are a pair of protuberances from the ventral surface of the female between the anus and the oviduct opening and that UGPs have a thick cortex of stratified epithlium as well as a highly vascularized medulla. The urinogenital papillae of the female medaka are protuberances between the anus and the genital pore. In the female, their anterior end is single and their posterior end is bi-lobed (rarely tri-lobed). They are persistent the year around although they seem to grow in size during the breeding season which, in Japan, extends from mid-April to mid-September.

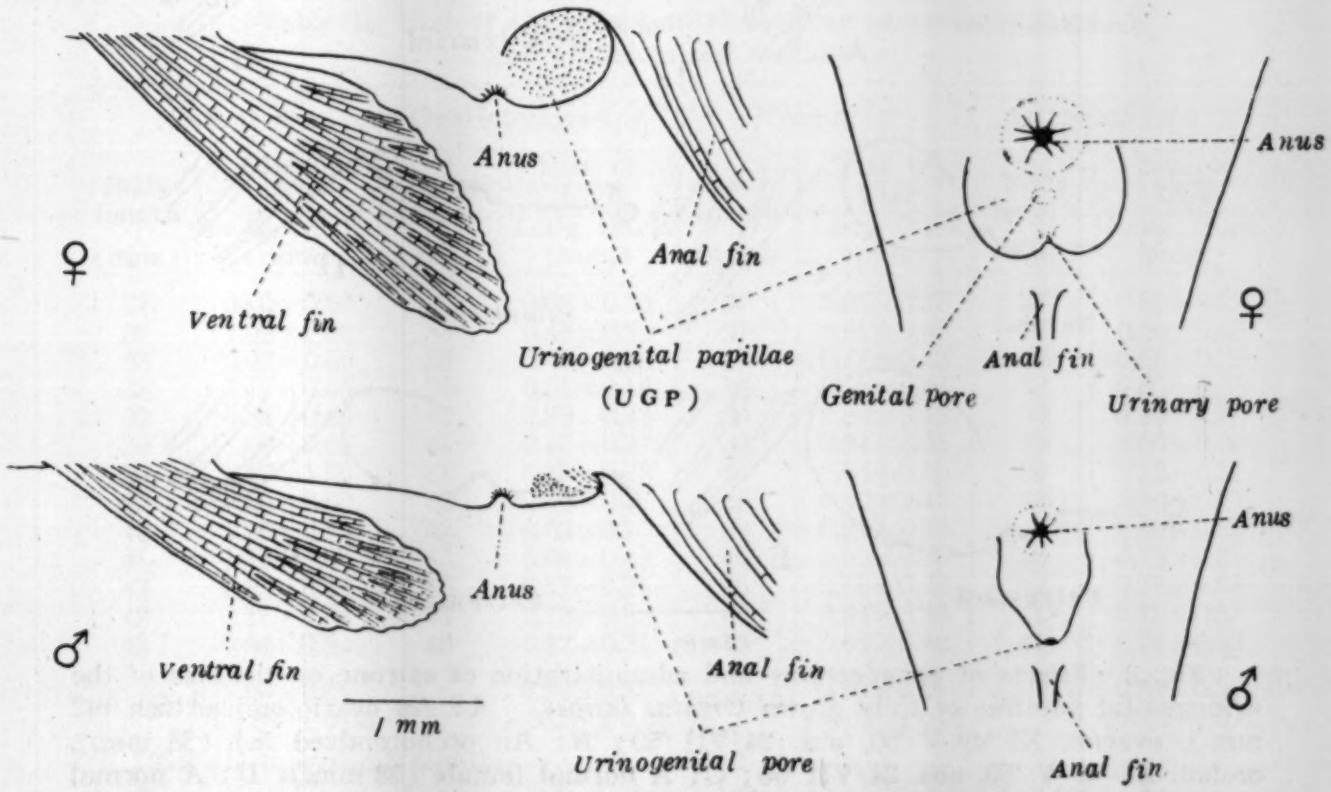

Fig. 1. Lateral (left) and ventral (right) views of the anal region of fully grown *Oryzias latipes*. In the left figures the medulla of the UGP is stippled.

The blood supply in the medulla becomes prominent during the summer months. The corresponding structure in the male is a small protuberance between the anus and the urinogenital pore. In the male it is uni-lobed in both anterior and posterior ends and its medulla is very small (Fig. 1).

Cross section through the UGPs of a full-grown female and a full-grown male are shown in Plate 7, *A* and *B*. The UGP of the medaka consists of two tissues, the cortex and the medulla. The cortex is non-vascularized and is nothing but a thickened epidermis which composed of ***stratum coreum*** and ***stratum germinativum***. At the base of the cortex is a basement membrane. The medulla is highly vascularized and it appears to be a modified mass of the corium. In the female a large medulla is surrounded by a thick epidermis which invade the medulla in places. In the male, on the other hand, the medulla is very small and the infiltrative process of the epidermis into the medulla is more pronounced than that in the female. In Plate 7 *B* is shown a cross-section of a normal male UGP in the non-infiltrative region. Pronounced infiltrative processes are present in other regions of the same preparation.

In passing it may be remarked that in the male the genital duct (*Vas deferens*) and the short urinary duct from the "urinary bladder" are fused at the end of the protuberance and open as the urinogenital pore, while in the female the oviduct and the urinary duct open separately. In fully-grown females the UGP cover both genital and urinary pores (Fig. 1).

It is obvious that the UGPs of the medaka are female-positive sex-charac-

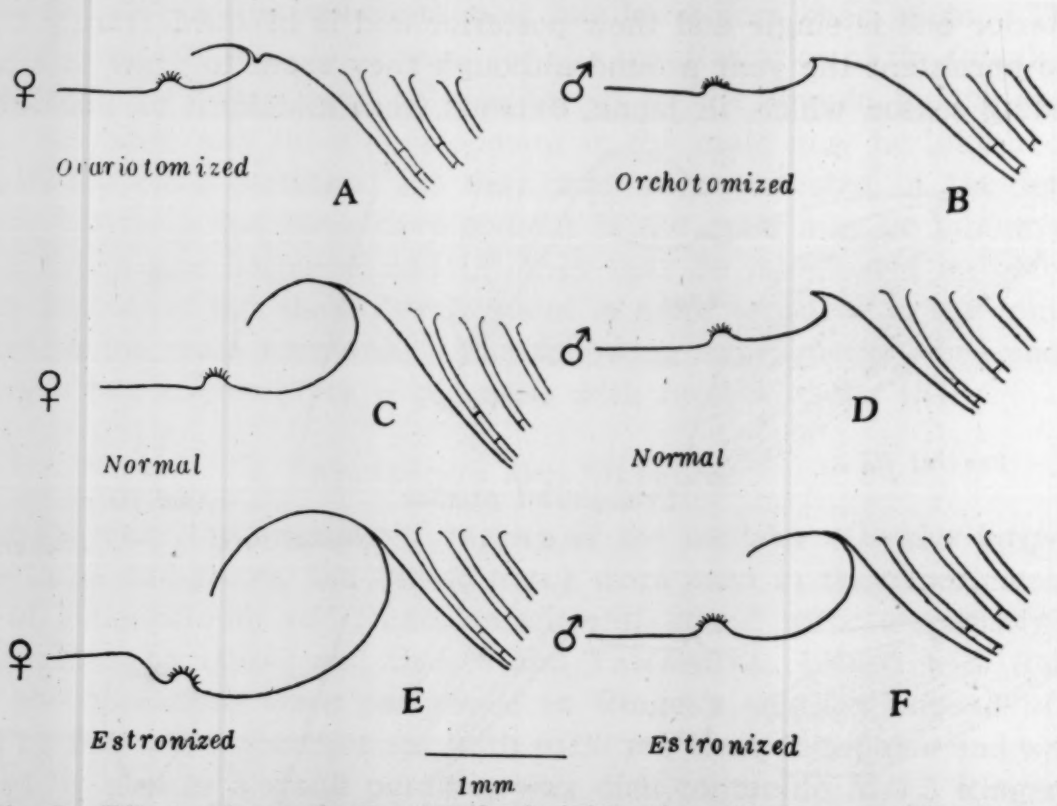

Fig. 2. Effects of gonadectomy and administration of estrone on the size of the urinogenital papillae of fully grown *Oryzias latipes*. *A*: An ovariotomized fish (42 mm.), ovariot. XI '49–V '50, obs. 24/VII '50; *B*: An orchotomized fish (38 mm.), orchot. XI '49–V '50, obs. 24/VII '50; *C*: A normal female (38 mm.); *D*: A normal male (37 mm.); *E*: An estrone-administered female (35 mm.), admin. 19/V '51, obs. 29/V '51; *F*: An estrone administered male (35 mm.), admin. 19/V '51, obs. 29/V '51.

ters in the sense that they are more developed in the female than the male. In young fish less than 16 mm. in length no sex-difference in external aspect is apparent although sex is already differentiated internally. Difference of the UGP in both sexes is recognizable when the fish are more than 16–17 mm., and in 20 mm. fish the difference is very prominent. The medulla of the UGP is noticeable as an orange-yellow mass in living young fish. When the young fish are almost 16–18 mm. in length the sex can be determined more easily by observing the UGP than by the differences in the anal and dorsal fins, which do not become prominent until the fish measures 20 mm. In the male the cleft of the dorsal fin and elongation of the anal fin are apparent at this stage. Papillar processes (PP) in the male anal-fin begin to appear when the fish is 22–26 mm. When they reach 26–28 mm. they attain sexual maturity.

Effect of Gonadectomy on Urinogenital Papillae (UGP)

Before going further it may be remarked that the gonad in this fish is a median, unpaired organ. The ovary is an unpaired sac-like organ filling most of the posterior body cavity. The testis is a slender organ between the peritoneum and the intestine.

Between November 1949 to May 1950, complete gonadectomy was performed on about eighty full-grown fish. Thirteen ovariotomized females and thirteen orchotomized males survived until the end of August 1950. Size of the UGP in both the gonadectomized fish and that of control fish were measured. The results are shown in Table 1 and typical cases are illustrated in Fig. 2. After ovariotomy the UGP in the females undergo remarkable reduction or atrophy.

Table 1. Size of the urinogenital papillae of ovariotomized and orchotomized medakas

Normal ♀		Ovariotomized ♀		Normal ♂		Orchotomized ♂	
Body length (mm.)	Size of UGP Leng. × thick. (mm.)	Body length (mm.)	Size of UGP Leng. × thick. (mm.)	Body length (mm.)	Size of UGP Leng. × thick. (mm.)	Body length (mm.)	Size of UGP Leng. × thick. (mm.)
37	0.50×0.60	37	0.66×0.33	37	0.65×0.43	37	0.66×0.25
37	1.00×0.66	38	0.46×0.33	37	0.81×0.43	37	0.66×0.40
38	0.93×0.66	38	0.43×0.30	37	0.77×0.43	38	0.66×0.26
38	1.17×0.65	39	0.31×0.19	38	0.65×0.43	38	0.63×0.36
39	0.84×0.65	40	0.52×0.43	39	0.84×0.43	39	0.88×0.33
39	1.00×0.65	41	0.42×0.27	39	0.84×0.65	39	0.60×0.30
39	1.17×1.00	41	0.39×0.23	39	0.64×0.40	39	0.73×0.37
39	1.00×0.60	42	0.50×0.17	39	0.50×0.40	39	0.60×0.33
40	1.00×0.84	42	0.52×0.27	39	0.84×0.43	40	0.66×0.43
41	1.17×0.65	43	0.66×0.43	40	0.50×0.50	40	0.73×0.31
42	0.84×0.65	43	0.57×0.25	40	0.56×0.43	40	0.71×0.35
42	1.17×0.84	43	0.78×0.53	40	0.84×0.50	41	0.72×0.37
45	0.84×0.84	43	0.52×0.31	43	0.65×0.40	41	0.75×0.33
40±0.4	1.00×0.71 ±0.02 ±0.02	41±0.5	0.52×0.31 ±0.05 ±0.02	39±0.3	0.70×0.45 ±0.02 ±0.02	39±0.2	0.69×0.33 ±0.01 ±0.01

UGP = Urinogenital papillae.

Ovariotomy and orchotomy were performed from Nov. 1949 to May 1950. Measurements were made at the end of Aug. 1950.

Both the length and the thickness are greatly reduced. The urinary pore of the female, which is covered by the UGP and is invisible in normal females, becomes visible in the ovariotomized fish.

The castration in the male, evokes no enlargement of the UGP and the length of the organ is not changed while its thickness tends to be reduced. This is due to the reduction of the medulla.

These results indicate that the presence and the maintenance of pronounced UGP in the female depend upon the presence of the ovary and that the removal of the testis in the male does not result in enlargement of the UGP. Poorly developed UGP in the male also is dependent on the presence of the testis.

Effect of Subcutaneous Administration of Estrone on Urinogenital Papillae (UGP)

The results of the preceding experiment of gonadectomy lead to the conclusion that manifestation and maintenance of the UGP in the female depend upon the function of the ovary. Administration of estrone to normal males and females was made in order to study the effect of the estrogen upon the development of the UGP. Pure powder of estrone was administered subcutaneously. The fish were anesthetized with chloretone and estrone powder was inserted between the scales by use of a needle. About 0.5 mg. of pure estrone was administered to each fish.

The subcutaneous administration of estrone brought about a remarkable enlargement of the UGP in both males and females (Table 2 and Plate 7, *C* and *D*). In May and June, during which the experiments were performed, the effect

Table 2. Effects of subcutaneous administration of estrone on the size of the urinogenital papillae in normal (full-grown) females and males

Females				Males			
Estrone		Control		Estrone		Control	
Body length (mm.)	Size of UGP Leng. × thick. (mm.)	Body length (mm.)	Size of UGP Leng. × thick. (mm.)	Body length (mm.)	Size of UGP Leng. × thick. (mm.)	Body length (mm.)	Size of UGP Leng. × thick. (mm.)
35	1.50×0.65	36	1.10×0.75	35	1.20×0.70	35	0.50×0.30
36	1.55×0.80	37	0.80×0.50	35	1.00×0.55	37	0.65×0.35
36	1.05×0.60	38	0.85×0.55	36	1.15×0.55	37	0.55×0.30
36	1.35×0.60	38	1.10×0.60	36	1.15×0.55	37	0.60×0.35
37	1.50×0.95	38	1.10×0.70	37	1.05×0.55	37	0.75×0.40
37	1.50×0.90	38	0.80×0.55	37	1.20×0.90	37	0.55×0.25
37	1.25×0.85	38	1.20×0.60	37	1.10×0.60	37	0.45×0.35
37	1.20×0.60	38	0.75×0.45	38	1.10×0.50	38	0.50×0.30
37	1.30×0.60	38	1.05×0.65	38	1.20×0.55	38	0.45×0.35
38	1.05×0.45	38	0.95×0.55	38	1.10×0.55	38	0.70×0.30
38	1.10×0.60	39	0.90×0.55	38	1.30×0.55	38	0.70×0.35
40	1.40×0.70	40	1.05×0.65	40	1.35×0.80	38	0.75×0.40
37±0.2	1.31×0.69 ±0.04 ±0.03	38±0.2	0.97×0.59 ±0.03 ±0.02	37±0.3	1.16×0.61 ±0.02 ±0.02	37±0.1	0.60×0.33 ±0.02 ±0.01

UGP = Urinogenital papillae.

Administration: 19/V '51, observation: 9/VI '51.

of estrone became apparent within a few days after administration. Administration of estrone to females induced remarkable hypertrophy or super-development of the UGP. In the males estrone induced the development of the UGP. Ten days after administration both the length and the thickness of the UGP in estronized male are doubled as compared with the control males, becoming slightly larger than the UGP of normal females. Bi-lobulation in the posterior region of the UGP, however, was not noted in estronized males. Typical cases of estronized UGP of males and females are illustrated in Fig. 2.

Cross sections through the UGP of both estronized female and male (Plate 7, *C* and *D*) indicate that it is the medulla rather than the cortex that shows hypertrophy under the influence of estrone. In both estronized females and males the infiltrative process of the cortex disappears because of enlargement of the medulla.

These experiments prove that the pronounced UGP in normal females are due to the presence of female hormone.

Effect of Subcutaneous Administration of Methyl Testosterone on UGP

As already mentioned, the UGP of the male also has the medulla although it is poorly developed. Castration brings about the reduction of the medulla. In order to elucidate the role of the male hormone in the manifestation of the

Table 3. Effects of subcutaneous administration of methyl testosterone on the size of the UGP in normal (full-grown) females and males

Females				Males			
Methyl testosterone		Control		Methyl testosterone		Control	
Body length (mm.)	Size of UGP Leng. × thick. (mm.)	Body length (mm.)	Size of UGP Leng. × thick. (mm.)	Body length (mm.)	Size of UGP Leng. × thick. (mm.)	Body length (mm.)	Size of UGP Leng. × thick. (mm.)
30	0.72×0.36	30	0.81×0.58	30	0.81×0.43	29	0.63×0.45
30	0.76×0.43	30	0.90×0.54	31	0.67×0.41	31	0.54×0.36
31	0.81×0.49	31	0.90×0.54	31	0.67×0.45	32	0.54×0.45
31	0.84×0.45	32	0.90×0.61	31	0.72×0.49	32	0.63×0.36
31	0.99×0.54	32	0.94×0.54	31	0.79×0.41	32	0.63×0.47
32	0.99×0.49	32	0.99×0.61	31	0.81×0.45	32	0.61×0.45
32	0.99×0.54	32	1.01×0.59	31	0.81×0.45	32	0.63×0.36
32	0.99×0.54	32	1.08×0.63	32	0.67×0.40	32	0.52×0.30
33	0.77×0.45	32	1.08×0.68	32	0.81×0.49	33	0.58×0.36
33	0.79×0.61	32	1.17×0.77	32	0.81×0.43	33	0.59×0.30
33	0.90×0.54	33	0.81×0.52	33	0.72×0.43	33	0.63×0.45
34	0.83×0.56	33	1.13×0.63	33	0.81×0.45	33	0.63×0.36
34	0.90×0.45	34	0.76×0.58	33	0.83×0.45	33	0.72×0.50
35	0.99×0.49	34	0.81×0.45	35	0.81×0.43	33	0.57×0.54
35	0.99×0.54	34	1.04×0.54	35	0.81×0.45	34	0.63×0.45
36	1.08×0.59	36	1.08×0.72	36	0.81×0.49	35	0.72×0.45
33±0.3	0.90×0.51 ±0.02 ±0.001	33±0.3	0.91×0.60 ±0.02 ±0.01	32±0.3	0.77×0.43 ±0.01 ±0.002	33±0.2	0.61×0.35 ±0.04 ±0.03

UGP = Urinogenital papillae.

Administration: 4/VIII '54, observation: 17/VIII '54.

medulla of the UGP in the male, the subcutaneous administration of methyl testosterone was made on normal males and females. Powder of methyl testosterone (about 0.5 mg.) was inserted between the scales with forceps. About two weeks after the administration the size of the UGP was measured. The results are shown in Table 3. In females no enlargement of the UGP was apparent while in the males a slight but significant enlargement of the UGP was noted. The effect of methyl testosterone is in contrast to that of estrone which induces profound hypertrophy of the UGP in both males and females. This indicates that the UGP is less sensitive to the androgen than to the estrogen. The result that methyl testosterone induced no enlargement in the normal female UGP may be interpreted to mean that the effect of the androgen was masked by a female hormone from the ovary although two hormones may act additively, since the female hormone has a more profound effect than the male hormone. This possibility appears to be valid because the period of administration was relatively short. In normal males in which the medulla is poorly developed the additive effects of both the administered androgen and the male hormone from the testis were apparent.

Effect of Transplantation of Ovary upon UGP in Castrated Males

If the UGPs are sexual characters depending upon female hormone for their development, it should be possible to induce development of the UGP in castrated males by implanting the ovary. Experiments of implantation of the ovary into castrated males have been performed in order to obtain further proof of the hormonal control of the UGP. Half of the ovary was implanted into the castrated male. Although the same operation was performed on fifty-six fish, all but one fish failed to survive. The successful case was given in Table 4. It is clear that there is an enlargement of the UGP which reached nearly the size of those of normal females.

Table 4. Effect of implantation of ovary on the urinogenital papillae in the castrated male

Date of measurement	Body length (mm.)	Size of urinogenital papillae: Length thickness (mm.)
2/VI	35	0.74×0.40
17/VI	35	0.74×0.40
31/VII	37	1.03×0.63

Castration: 2/V '51, transplantation of ovary: 2/V '51.

Effect of Estrone upon UGP in Ovariotomized Fish

From the preceding experiments it might be expected that the atrophied UGP in ovariotomized fish would begin to develop by the administration of estrone. Ovariotomy was performed on fully grown females at the end of April 1951. By the middle of June 1951 the retrogession of the UGP became apparent. Then

Table 5. Effect of subcutaneous administration of estrone on the urinogenital papillae in the ovariotomized medakas

No. of fish	Before administration (14/VI '51)		After administration (24/VI '51)	
	Body length (mm.)	Size of urinogenital papillae Length thickness (mm.)	Body length (mm.)	Size of urinogenital papillae Length thickness (mm.)
1	33	0.85×0.50	33.5	1.10×0.80
2	〃	0.90×0.50	〃	1.25×0.80
3	〃	0.70×0.55	〃	1.12×0.80
4	34	0.75×0.45	34.5	1.30×0.85
5	〃	0.70×0.45	〃	1.15×0.75
Average	34.4	0.78×0.49	33.9	1.18×0.80

Ovariotomy: 23-26/IV '51, administration of estrone: 14/VI '51, observation: 24/VI '51.

pure estrone was administered subcutaneously on the spayed fish. The results are shown in Table 5. It will be seen that estrone induces an enlargement of the UGPs which are retrogressing because of the absence of the ovary. The size of the UGP became more pronounced than that in normal females. This experiment affords a further proof that estrone exerts an obvious influence upon development of the UGP.

Discussion

It is true that in most cases the positive sex-characters of fish are on the male side. Sexual dimorphism in many fish is expressed by presence of some structures in the male and absence of those in the female or by a more pronounced structures in the male while there is less pronounced ones in the female. These sex-characters may be called the male-positive characters. Recent reports by investigators in this field show conclusively that the manifestations of these male-positive sex-characters are dependent upon the male hormone. Experiments performed by Nagata (1934, 1936) and Okada and Yamashita (1944 *a*) in the medaka are also concerned with male-positive characters, such as the presence of papillar processes (PP) in the anal fin of the male. The gonopodium in the male of viviparous cyprinodonts, may also regarded as a male-positive character since it is differentiated from the immature anal fin. The anal fin of the female in these fish has the pattern of the immature anal fin. Investigators in viviparous cyprinodonts (*loc. cit.*) confirmed that the gonopodium is dependent upon the presence of male hormone in its differentiation. These facts lead to some authors to believe that external sex-characters of the male are differentiated from the female pattern under the influence of male hormone and that the ovary exerts no influence upon the external sex-characters of fish. In a number of less known cases, the female has something definite and positive which the male has not or if he has, it is in a more indistinct form. These structures may be called female-positive sex-characters. The ovipositor of female bitterling is a typical example. Fleischmann and Kann (1932, 1934, 1937) proved that estrogens induce

elongation of the ovipositor in the female bitterling (*Rhodeus amarus*) in non-breeding seasons. According to KLEINER *et al.* (1936, 1937, 1940) elongation of the ovipositor in the female bitterling is also induced by androgens, progesterone and adrenal extract. FLEISCHMANN and KANN (1938) proved that corticosterone will also give a positive result.

The urinogenital papillae of the medaka belong to the female-positive sex-characters since they are pronouncedly developed in the female and the corresponding structure in the male is poorly developed.

There are three possibilities to account for the manifestation of this female-positive sex-character. The first possibility is that its development in the male is inhibited by the male hormone and that it develops pronouncedly in the female because the absence of the male hormone. Since the UGP dose not enlarge after castration in males, this possibility is untenable. The second possibility is that it may be produced in the female by the female hormone and it is indistinct in the male because of the absence of the female hormone. Although this possibility is partially true, it can not account for the presence of poorly developed medulla in the UGP in the male and its reduction after castration. The third possibility is that the medulla of the UGP is manifested both by the female and male hormones but its development is far more sensitive to the action of the female hormone than to the male hormone. The last seems to be the most plausible. In the normal female the UGP is pronouncedly developed by the female hormone produced by the ovary. In the male it is poorly developed by the male hormone from the testis which has only a limited effect in the manifestation for the UGP.

In this connection it may be remarked that sex-reversed females of the male genotype (XY) in the medaka, the sex-reversal of which was induced through the action of estrogens from the hatching fry to the stage of several months, have developed typical urinogenital paillae of the female type (YAMAMOTO 1953). The UGPs of functional sex-reversals were persistent after the cessation of the oral administration of estrogens (estrone or diethyl stilbestrol). In this case the UGP of fish during the period of estrogen-administration might be manifested by the additive effect of administered estrogen and the female hormone from the reversed gonad (ovary). After the cessation of estrogen-administration the UGP of the sex-reversed females of the male genotype might be retained by the action of the ovarian hormone.

In higher vertebrates an ambisexual hormonal production has been known. The female produces a large amount of female hormone as well as a small amount of male hormone while the reverse is true in the male. There is the possibility that the pronounced UGP in the female is dependent upon the large amount of the female hormone plus the small amount of the male hormone and that the poorly developed UGP in the male depends on a large amount of the male hormone plus a small amount of the female hormone. Although this possibility is quite in harmony with the experiments mentioned above, the ambisexual hormone-production in fish has not yet been explored.

Summary

1. The urinogenital papillae of the female medaka (*Oryzias latipes*) are fleshy bi-lobed protuberances from the ventral surface between the anus and the oviduct opening. In the male, the corresponding structure is present in a poorly developed state between the anus and the urinogenital pore. The urinogenital papilla consists of consists of the cortex and the medulla. It is the medulla that is pronouncedly developed in the female. Therefore the urinogenital papilla may be regarded as a female-positive external sex-character.

2. The ovariotomy induces an atrophy of the medulla of the urinogenital papillae. The castration in the male also reduces the medulla. Implantation of the ovary into castrated male causes development of the organ.

3. Retrogressing urinogenital papillae in ovariotomized females undergo hypertrophy by the administration of estrone.

4. In both sexes subcutaneous administration of estrone on normal fish induces hypertrophy of the urinogenital papillae. Subcutaneous administration of methyl testosterone in the normal male induces a slight but significant enlargement of the urinogenital papilla.

5. It is concluded that the medulla of the urinogenital papilla is manifested by both the female and male hormones but its development is far more sensitive to the female hormone than to the male hormone.

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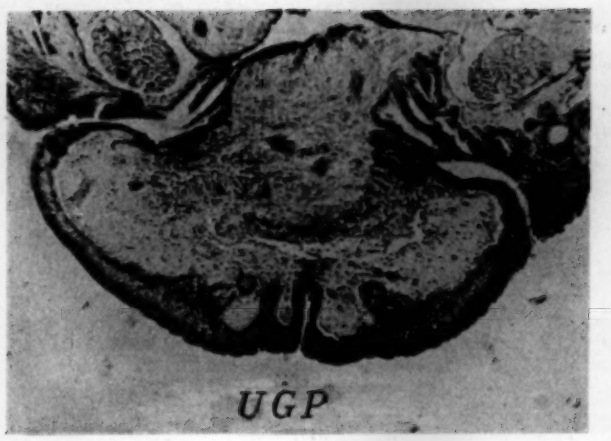
Literature

BALDWIN, F. M. and GOLDIN, H. S., 1940. Effects of testosterone propionate on the female viviparous teleost, ***Xiphophrous helleri*** Heckel. Proc. Soc. Exp. Biol. and Med., *42*, 813–819. — EVERSOLE, W. J., 1939. The effects of androgens upon the fish (***Lebistes reticulatus***). Endocrinology, *25*, 328–330. — FLEISCHMANN, W. und KANN, S., 1952. Über eine Funktion des weiblichen Sexualhormons bei Fischen (Wachstum der Legeröhre des Bitterlings). Pflüger's Arch., *230*, 662-667. — FLEISCHMANN, W. und KANN, S., 1934. Über das Wachstum der Legeröhre des Bitterlings unter dem Einfluss des weiblichen Sexualhormons. II. Pflüger's Arch., *235*, 130-136. — FLEISCHMANN, W. und KANN, S., 1937. Über das Wachstum der Legeröhre des Bitterlings unter dem Einfluss des weiblichen Sexualhormons. Pflüger's Arch., *238*, 711-712. — FLEISCHMANN, W. und KANN, S., 1938. The bitterling ovipositor reaction to corticosterone. Science, *87*, 305–306. — GROBSTEIN, C., 1940. Effect of testosterone propionate on regenerating anal fin of adult ***Platypoecilus maculatus*** females. Proc. Soc. Exp. Biol. and Med., *45*, 484–486. — GROBSTEIN, C., 1942. Endocrine and developmental studies of gonopod differentiation in certain poeciliid fishes. II. Effect of testosterone propionate on the normal and regenerating anal fin of adult ***Platypoecilus maculatus*** females. J. Exp. Zool., *89*, 305–828. — HILDEMANN, W. H., 1954. Effects of sex hormones on the secondary sex characters of ***Lebistes reticulatus***. J. Exp. Zool., *126*, 1–15. — HOPPER, A. F., 1949 ***a***. Development and regeneration of the anal fin of normal and castrated males and females of ***Lebistes reticulatus***. J. Exp. Zool., *110*, 299–318. — HOPPER, A. F., 1949 ***b***. The effect of ethynyl testosterone on the intact and regenerating anal fins of normal and castrated females and normal males of ***Lebistes reticulatus***. J. Exp. Zool., *111*, 393–414. — KLEINER, I. S., 1940. The relative effects of progesterone

and testosterone propionate upon ovipositor lengthening of the female bitterling. Endocrinology, *26*, 534–535. — KLEINER, I. S., WEISMANN, A. I. and MISHKIND, D. I., 1936 *a*. Use of the female bitterling as a test for male hormone. J. Amer. Vet. Med. Ass., *106*, 1943. — KLEINER, I. S., WEISMANN, A. I. and MISHKIND, D. I., 1936 *b*. Effect of crystalline synthetic androsterone on the female bitterling. Proc. Soc. Exp. Biol. and Med., *35*, 344–345. — KLEINER, I. S., WEISMANN, A. I. and MISHKIND, D. I., 1937. The similarity of action of male hormones and adrenal extracts on the female bitterling. Science, *85*, 75. — NAGATA, Y., 1934. Gonadectomy in Medaka (Japanese). Zool. Mag. (Japan), *46*, 293–294. — NAGATA, Y., 1946. Transplantation of testis into ovariotomized *Oryzias latipes* (Japanese). Zool. Mag. (Japan), *48*, 102–108. — OKA, T. B., 1931. On the processes on the fin-rays of the male of the male of *Oryzias latipes* and other sex characters of this fish. J. Fac. Sci. Imp. Univ. Tokyo, Sec. IV (Zool.), *2*, 209-218. — OKA, T. B., 1938. Guanophores in fins of male medaka (secondary sexual character)(Japanese), Zool. Mag. (Japan), *50*, 173-174. — OKADA, YÔ K., 1943. A review of literature on the secondary sexual characters in fish and their experimental consideration (Japanese). Ann. Rev. of Exp. Morph. (Tokyo), *1*, 34-64. — OKADA, YÔ K. and YAMASHITA, H., 1944 *a*. Experimental investigation of the manifestation of secondary sexual characters in fish, using the medaka, *Oryzias latipes* (Temminck and Schlegel) as material. J. Fac. Sci., Tokyo Imp. Univ., sec. IV (Zool.), *6*, 383–437. — OKADA, YÔ K. and YAMASHITA, H., 1944 *b*. Experimental investigation of the sexual characters of poeciliid fish. J. Fac. Sci. Imp. Univ. Tokyo, sec. IV (Zool.), *6*, 589–633. — REGNIER, M., 1938. Contribution a l'etude de la sexualité des Cyprinodont vivipares (*Xiphophorous helleri, Lebistes reticulatus*). Bull. Biol., *72*, 285–493. — ROBINSON, E. J. and RUGH, R., 1943. The reproductive processes of the fish, *Oryzias latipes*. Biol. Bull., *84*, 115-125. — TURNER, C. L., 1941. Gonopodial characteristics produced in the anal fins of females of *Gambusia affinis affinis* by treatment with ethinyl testosterone. Biol. Bull., *80*, 371-383. — WITSCHI, E. and CROWN, E. W., 1937. Hormones and sex determination in fishes and in frogs. Anat. Rec., *70* (Suppl.), 121. — YAMAMOTO, T., 1953. Artificially induced sex-reversal in genotypic males of the medaka (*Oryzias latipes*). J. Exp. Zool., *123*, 571-594.



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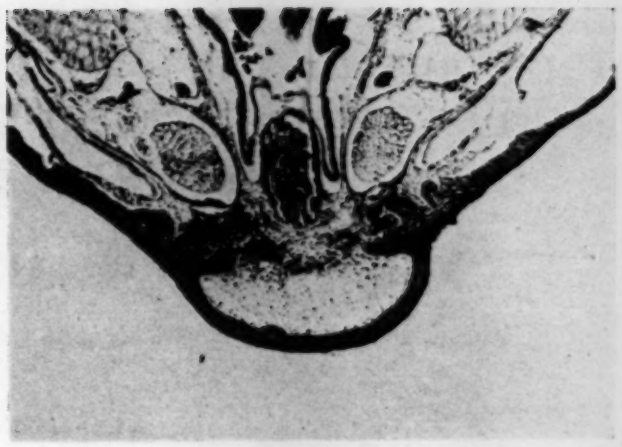

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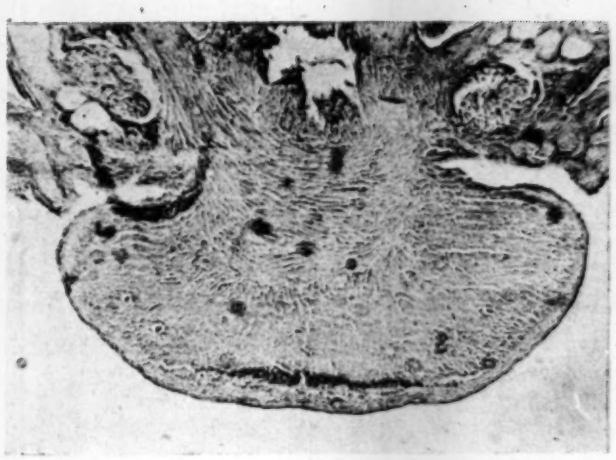

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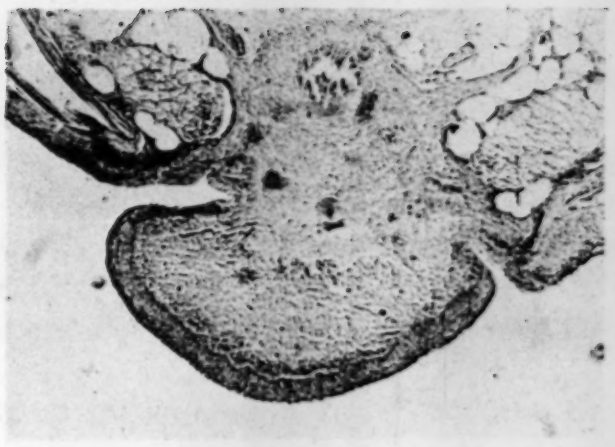

D

A: A cross-section through the region of the urinogenital papillae (UGP) of a normal female, the body length of which was 40 mm. (caudal included). Fixation: Apr. 27, 1951.

B: A cross-section through the region of the urinogenital papillae (UGP) of a normal male, the body length of which was 37 mm. (caudal included). Fixation: Apr. 27, 1951.

C: A cross-section through the region of the urinogenital papillae (UGP) of an estrone-administered female, the body length of which was 38 mm (caudal included). Three weeks after the subcutaneous administration of estrone. Fixation: Jun. 9, 1951.

D: A cross-section through the region of the urinogenital papillae (UGP) of an estrone-administered male, the body length of which was 38 mm. (caudal included). Three weeks after the subcutaneous administration of estrone. Fixation: Jun. 9, 1951.

INDUCTIVE EFFECT OF SOME FRACTIONS OF TISSUE EXTRACTS AFTER REMOVAL OF PENTOSE NUCLEIC ACID, TESTED ON THE ISOLATED ECTODERM OF *TRITURUS*-GASTRULA[1]

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Introduction

Regionally characterized structures were induced by pieces of various tissues of adult animals when implanted in the blastocoel or in the isolated ectoderm of *Triturus*-gastrula (Holtfreter, 1934; Chuang, 1938, 1939, 1940; Toivonen, 1940, 1945, 1953; Fujii, 1941, 1944; Rotmann, 1942; Hama, 1944; Kawakami and Kawakami, 1947; Okada, 1948). In addition, it has been found that some extracts of tissues possess strong regional inductive ability (Toivonen and Kuusi, 1948; Toivonen, 1949, 1950; Yamada, 1950*b*). Using the isolated ectoderm of *Triturus pyrrhogaster* as the reacting material, the chemical nature of the inducing factors in the kidney was analysed at our laboratory. It was then established that the tissue extracts rich in pentose nucleic acid (PNA) retained the characteristic inducing ability of the original tissue (Yamada, 1952; Yamada and Takata, in press; Hayashi, unpublished).

To determine the significance of PNA in the induction by these tissue extracts, researches were made along two lines. In the first group of experiments, PNA separated from guinea pig kidney extract was tested on the isolated ectoderm for its inductive ability. In the second group, PNA was removed from fractions of guinea pig kidney extracts and from rat liver extract by treating them with crystalline ribonuclease, and the inductive effect of those treated fractions of extracts was tested in the same manner as in the first group. The results of the first group of experiments have already been published (Yamada, Takata and Osawa, 1954), and in the present paper the second group of experiments are reported.

Material and Methods

The extracts of guinea pig kidney or rat liver containing pentose nucleoprotein were incubated with crystalline ribonuclease and desoxyribonuclease (Worthing-

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ton Biochemical Sales Co.) at room temperature for 6–8 hours. During incubation, the mixture was dialyzed against the buffer solution or buffered saline solution used for extraction, supplemented by magnesium salt, and kept in constant motion on a shaker. After incubation, proteins in the treated extracts were precipitated by ammonium sulphate. The proteins thus precipitated were partly or almost completely free from PNA. After removing ammonium sulphate through dialysis, these protein fractions were preserved in cold 95% ethanol. They were tested for their inductive ability within 5 days after preparation, because longer preservation might have affected their regional effect of induction (ENGLÄNDER, JOHNEN and VAHS, 1953; YAMADA and TAKATA, in press). Technical details for preparing protein samples implanted in each experimental series are described in the corresponding sections.

PNA contents of these protein samples were checked by measuring their ultraviolet absorption at 260 mμ with Beckman's spectrophotometer following SCHNEIDER's method (1945), and their nitrogen contents were measured according to LEVY and PALMER (1940). PNA contents were expressed by PNA-P μg/mg N. The contents of desoxypentose nucleic acid were measured by the method of CERIOTTI (1952). However, desoxypentose nucleic acid could not be detected in every sample prepared.

In all series of experiments, a piece of protein samples preserved in cold 95% ethanol was enclosed in two pieces of the presumptive ectoderm of early gastrula of *Triturus pyrrhogaster* after thorough washing in sterile HOLTFRETER's solution. Pieces of presumptive ectoderm were isolated according to the procedure described by YAMADA (1950 *b*). The explants so obtained were cultured at 18° C. for 9–14 days in sterile HOLTFRETER's solution adjusted to pH 7.2–7.3 by addition of sodium bicarbonate. After culturing, the explants were fixed in BOUIN's fluid, sectioned at 10–11 μ, stained with MAYER's haemalum and picro-blauschwarz and examined microscopically. On microscopic examination, structures induced in the explants were judged according to the criteria adopted at our laboratory, which have been published in part in papers of YAMADA (1950 *b*) and YAMADA and TAKATA (in press).

EXPERIMENTS

A. Control Experiments of Reaction System (Series CR).

The presumptive ectoderm was isolated from early gastrula of *Triturus pyrrhogaster* before coming into contact with the invaginating endo-mesoderm. Two pieces of ectoderm thus isolated were fused by putting together their two internal surfaces. Forty-three explants were prepared and cultured at 18°C. for 9 days in sterile HOLTFRETER's solution adjusted to pH 7.2–7.3 by sodium bicarbonate. All explants differentiated into aggregates of epidermal cells with a characteristically wrinkled surface and no neuralized cells could be recognized among them (Table I). These results confirmed earlier experiments of YAMADA (1950 *a, b*), and showed that in the species used and under the present conditions, the presumptive ectoderm does not have a tendency to differentiate neural or mesodermal structures.

B. Experimental Group I.

Preparation of the samples used in Experimental Series PH and con PH:

A pair of guinea pig kidneys (6.3 g.) was homogenized in 20 cc. of N/10 phosphate buffer (pH 6.1) at 0° C. and the homogenate was kept at 5° C. for 30 minutes. Then, it was centrifuged for 20 minutes at 0° C. and 3,500 r.p.m. The pH of the extract so obtained was 6.35. To 7 cc. of this supernatant were added 1 cc. each of ribonuclease solution (1 mg/cc.), desoxyribonuclease solution (1 mg/cc.) and M/10 $MgSO_4$ solution, all of which had been dissolved in the same phosphate buffer. Ten cc. of the mixture was incubated at 20° C. for 6 hours under dialyzing and shaking, as mentioned previously. After incubation, an equal volume of ammonium sulphate solution saturated at 5° C. (pH 5.45) was added to the treated extract and the mixture was kept at 5° C. for 30 minutes. Then, the precipitate was centrifuged down at 0° C., collected and dissolved in the phosphate buffer (N/10, pH 6.1) and dialyzed in a cellophane bag against the same buffer solution at 5° C. for 40 hours with frequent renewals of the external medium. After making sure of the complete removal of ammonium sulphate by NESSLER's reagent, cold 95% ethanol was added to the dialyzed solution up to the final concentration of 75%. Centrifuged down at 0° C., the precipitate was transferred to cold 95% ethanol and preserved at 5° C. This sample was designated as PH, and contained 9.0 PNA-P μg/mg N.

As a control for this preparation, the incubation mixture was prepared differing from the experimental mixture only in that the enzyme solutions to be added had been boiled for one hour. The incubation was carried out under the same conditions, and a protein fraction was obtained as described in the preparation of PH. This sample was designated as con PH, and contained 21.1 PNA-P μg/mg N.

Table I. The inductive ability of a protein fraction of phosphate extract of the kidney and the effect of partial removal of PNA (Experimental Group I).

Experimental Series	Number of available explants	Number of explants with any induction	Number of explants including induction types:													
			Somite	Notochord	Spinal cord	Deuterencephalon	Ear vesicle	Eye	Archencephalon	Nose	Lense	Pigment vesicle	Mesenchyme	Melanophore	Indifferent brain, or neural epithelium	Neural fragment
CR	43	0														
PH	54	54	31	5	17	41	34	1	0	0	0	0	38	24	33	4
Con PH	52	50	16	1	11	37	34	0	0	0	0	0	43	18	30	1

CR: Control experiments of reaction system, culture of isolated presumptive ectoderm. *PH:* Implantation of a protein fraction of phosphate extract of guinea pig kidney, precipitated by one-half saturation of ammonium sulphate after nuclease treatment. PNA content, 9.0 PNA-P μg/mg N. *Con PH:* Implantation of the same protein fraction without nuclease treatment. PNA content, 21.1 μg/mg N.

Results of Experimental Series PH: Deuterencephalic and spino-caudal structures were induced in all the explants except two; out of 54 explants with induced structures, 44 explants showed deuterencephalic and 26 showed spino-caudal character (Figs. 1 and 2). In one of these two exceptions, neural epithelium and

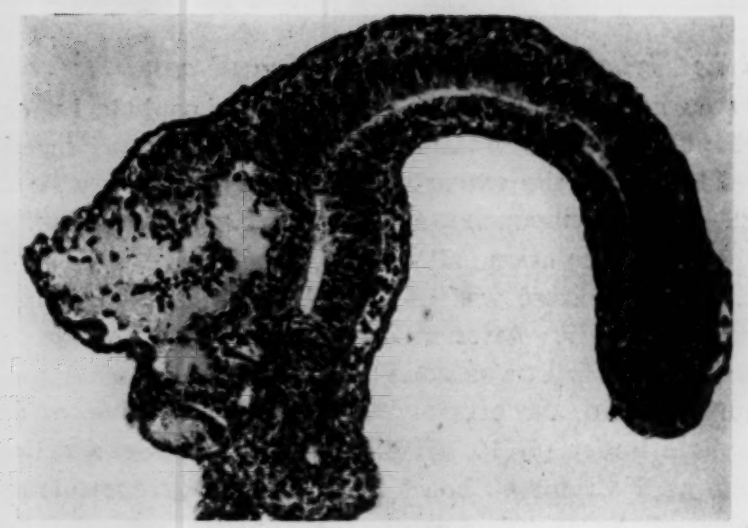

Fig. 1

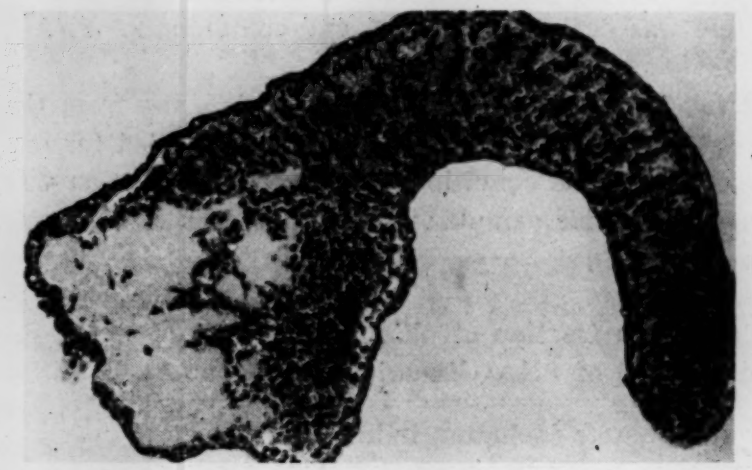

Fig. 2

Fig. 3

Fig. 1 and 2. A spinal cord-type structure (Fig. 1) accompanied by serially arranged somite-type structure (Fig. 2), induced by a nuclease-treated protein fraction of phosphate extract of guinea pig kidney (Series PH in Exp. Group I). On the left the implant (× 60).

Fig. 3. A deuterencephalon-type structure, accompanied by ear vesicle-type structure, induced by the same protein fraction not treated with nucleases (Series con PH in Exp. Group I). On the left the implant (× 60).

mesenchyme cells were induced without any clear regional character. In the other explant were induced neural epithelium and small eye-tye structure with accumulated pigment, manifesting archencephalic character (Tables I and V).

Results of Experimental Series con PH: The sample also induced deuterencephalic or spino-caudal structures (Fig. 3); out of 52 explants 40 were deuterencephalic and 18 were spino-caudal (Tables I and V). Accordingly, neither the inductive frequency nor the regionality of induction suffered any significant change through the reduction of PNA content of the implant.

C. Experimental Group II.

Preparation of the samples, AC, AC-1, con AC-1, AC-2, and con AC-2:

A pair of guinea pig kidneys (4.0 g.) was homogenized at 0° C. with 20 cc. of acetate buffer (N/10, pH 6.2) and extracted by exactly the same manner as in Series PH. The pH of this acetate extract was 6.45. To a small aliquot of this extract, cold 95% ethanol was added up to the final concentration of 75%, and the precipitate was centrifuged down at 0° C. and transferred to 95% ethanol of 5° C. ready for use. This ethanol-precipitate containing 7.4 PNA-P μg/mg N was designated as AC.

Two incubation mixtures were prepared by adding 1 cc. of M/10 $MgCl_2$ solution to each 7 cc. of the acetate extract; one with the nuclease solutions of the same concentration as in

Series PH and the other with the boiled nuclease solutions. They were incubated for 6 hours at 21°–23° C. Simultaneously they were shaken and dialyzed against N/10 acetate buffer of pH 6.2 supplemented by $MgCl_2$ in the same concentration as the incubated mixtures. After incubation, an equal volume of $(NH_4)_2SO_4$ solution saturated at 5° C. and adjusted to pH 6.0 was added to each of the incubated extracts, and the mixtures were kept at 5° C. for 30 minutes. The precipitates were centrifuged down at 0° C. and dialyzed at 5° C. in cellophane bags against N/10 acetate buffer of pH 6.2 for 48 hours. After completely removing $(NH_4)_2SO_4$, proteins were precipitated by cold ethanol, collected and preserved as in Experimental Group I. The sample obtained after nuclease digestion was designated as AC-1 and found to contain 0.9 PNA-P μg/mg N. The control sample without nuclease digestion was designated as con AC-1 and found to contain 7.9 PNA-P μg/mg N.

Further, a pair of kidneys (3.9 g.) was homogenized and extracted with 20 cc. of the same acetate buffer as in AC-1. An aliquot of 7 cc. extract was treated with nucleases of twice the concentration of that in AC-1, and an aliquot of 9 cc. was incubated without nuclease solutions, both supplemented by 1 cc. of M/10 $MgCl_2$ solution. The incubation was carried

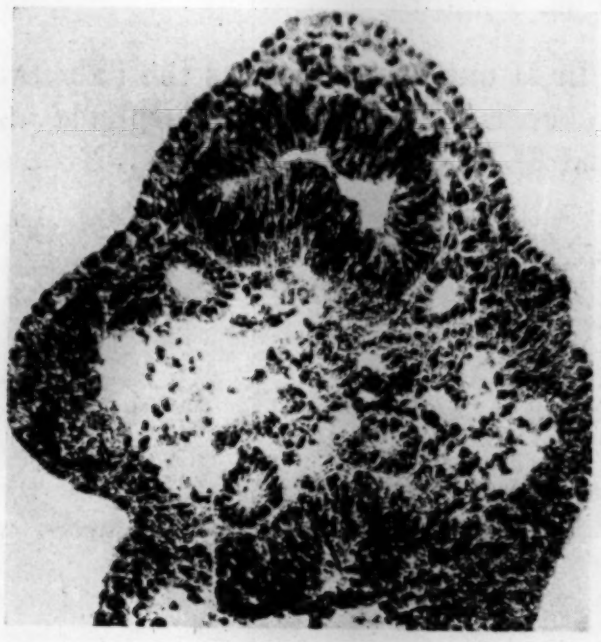

Fig. 4

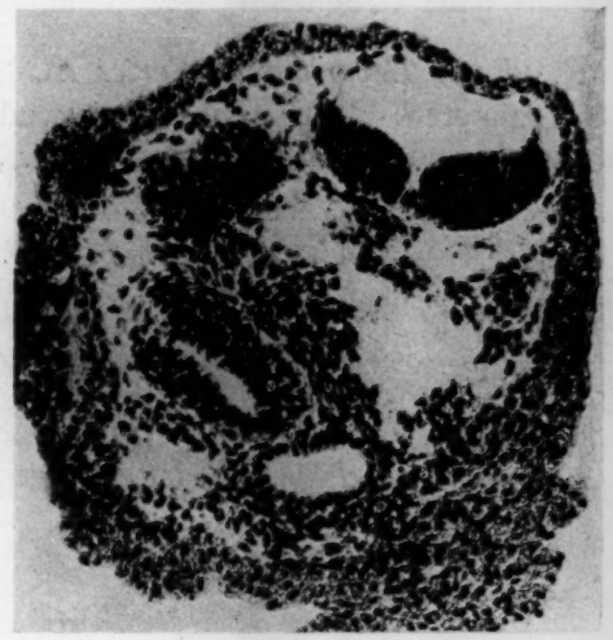

Fig. 5

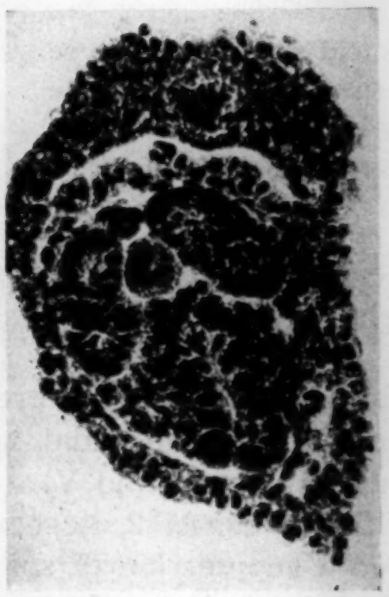

Fig. 6

Fig. 4. A strongly winding neural vesicle accompanied by several ear vesicle-type structures and mesenchyme, induced by a protein fraction of acetate extract of guinea pig kidney treated with nucleases (Series AC-1 in Exp. Group II, × 60).

Fig. 5. A section showing a deuterencephalon-type structure with an ear vesicle-type structure and mesenchyme, induced by the same protein fraction not treated with nucleases (Series con AC-1 in Exp. Group II). The implant in the middle (× 75).

Fig. 6. A transverse section through a tail-like structure induced by the same sample as that used in Fig. 5. The notochord-type (*middle*), accompanied by somite-type (*both sides*) and spinal cord-type (*below*). Note the formation of a "fin" (× 110).

out at 21°-23° C. for 8 hours in the same manner as described above. Two samples to be implanted were prepared through exactly the same procedure as in AC-1; one with nuclease treatment was designated as AC-2 and the other without treatment as con AC-2. Estimation of PNA content gave values of 0.7 PNA-P μg/mg N and 6.2 PNA-P μg/mg N, respectively.

Results of Experimental Series AC: Out of 47 explants, 5 contained archencephalic, 32 deuterencephalic and 5 spino-caudal inductions while 8 explants could not be regionally specified (Tables II and V).

Results of Experimental Series AC-1: All of 50 explants showed inductions. Three archencephalic, 44 deuterencephalic and 5 spino-caudal explants together with 4 without any regional character could be identified (Fig. 4).

Results of Experimental Series con AC-1: Out of 32 explants, 30 showed inductions; 2 archencephalic, 25 deuterencephalic and 4 spino-caudal explants together with 4 regionally unspecifiable (Figs. 5 and 6).

Results of Experimental Series AC-2: In 41 out of 46 explants the induction was registered. Ten explants showed archencephalic, 33 deuterencephalic and only one spino-caudal character (Figs. 7 and 8).

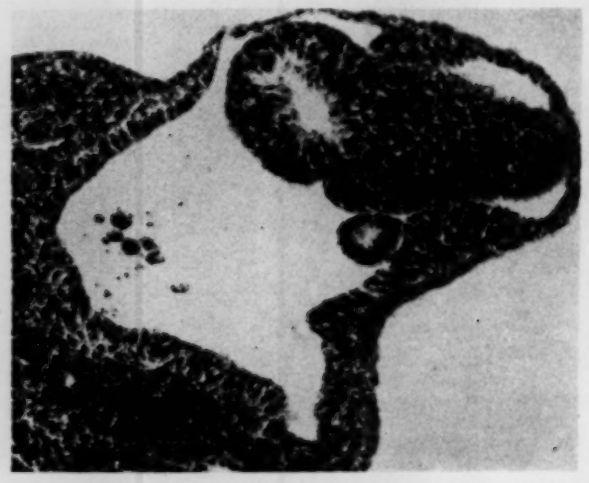

Fig. 7

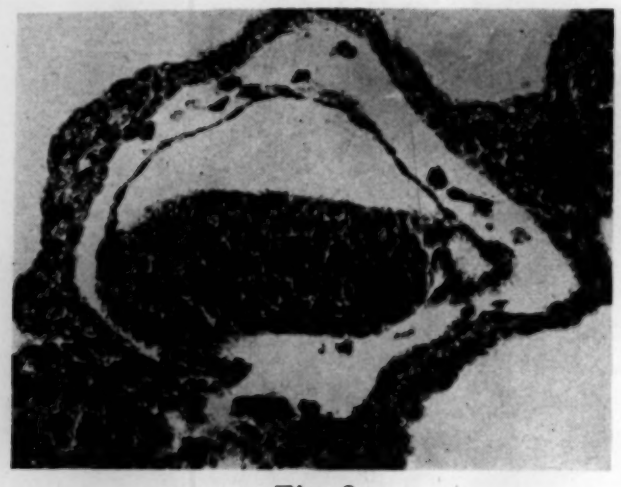

Fig. 8

Fig. 7. A brain-like structure accompanied by a lentoid and two nose-type structures on both sides, induced by a nuclease-treated protein fraction of acetate extract of guinea pig kidney (Series AC-2 in Exp. Group II, × 70).

Fig. 8. Another explant of the same series. A deuterencephalon-type structure induced with some mesenchyme cells (× 90).

Results of Experimental Series con AC-2: Thirty-eight out of 39 explants showed induction. Among these explants, 2 archencephalic, 32 deuterencephalic and 8 spino-caudal inductions were distinguished.

Thus all the samples in this experimental group induced principally deuterencephalic structures in the larger number of the explants and spino-caudal and archencephalic structures in a small number of explants (Tables II and V).

Comparing the results of Series AC-2 with those of Series con AC-2, it seems that the reduction of PNA content of the implant caused a suppression of spino-caudal effect, with a complementary increase of the archencephalic tendency.

Table II. The inductive ability of a protein fraction of acetate extract of the kidney and the effect of extensive removal of PNA (Experimental Group II).

Experimental Series	Number of available explants	Number of explants with any induction	Number of explants including induction types: Somite	Notochord	Spinal cord	Deuterencephalon	Ear vesicle	Eye	Archencephalon	Nose	Lens	Pigment vesicle	Mesenchyme	Melanophore	Indifferent brain, or neural epithelium	Neural fragment
AC	47	43	3	0	4	29	13	0	4	1	2	3	35	34	17	21
AC-1	50	50	2	0	1	42	26	0	1	1	0	1	35	26	23	22
Con AC-1	32	30	4	2	2	19	17	1	1	0	2	0	19	9	12	11
AC-2	46	41	3	0	1	30	13	0	9	2	4	5	34	19	19	11
Con AC-2	39	38	7	0	6	31	17	0	2	0	1	1	32	25	31	7

AC: Implantation of ethanol-precipitate of acetate extract of guinea pig kidney, containing 7.4 PNA-P μg/mg N. *AC-1:* Implantation of a protein fraction of guinea pig kidney, precipitated by one-half saturation of ammonium sulphate after a nuclease treatment. PNA content, 0.9 PNA-P μg/mg N. *Con AC-1:* Implantation of the same protein fraction without nuclease treatment. PNA content, 7.9 PNA-P μg/mg N. *AC-2:* Implantation of a protein fraction similar to AC-1, but with higher concentration of the enzymes. PNA content, 0.7 PNA-P μg/mg N. *Con AC-2:* Implantation of the same protein fraction of acetate extract without nuclease digestion. PNA content, 6.2 PNA-P μg N.

However, when the figures obtained were examined statistically, the apparent difference in regional effect between AC-2 and con AC-2 was untenable at the level of significance of 5%. Accordingly, as judged by the frequency and the regional character of induction, marked reduction of PNA content of the implant did not cause significant change in inductive effects on the ectoderm explants in the present experimental group.

D. Experimental Group III.

Preparation of the samples, Nase-A and con Nase-A:

It is well established that ribonuclease does not completely digest PNA (ZITTLE, 1946; LORING, CARPENTER and ROLL, 1947). Then, for obtaining PNA-free protein fractions from solutions containing pentose nucleoprotein through the application of ribonuclease, the conditions must be avoided that precipitate undigested "ribonuclease-resistant fraction" of PNA together with proteins. This is accomplished by adjusting pH of the mixtures to 7.0 when proteins are precipitated by ammonium sulphate.[3)]

[3)] The author is indebted to Dr. YASUO YAGI for suggesting this excellent technique. That PNA remained in the samples after nuclease digestion in Experimental Series PH, AC-1 and AC-2 may be explained as due to concomitant precipitation of undigested PNA together with proteins in acidic media.

As shown in Experimental Group II, the marked spino-caudal effect of the original guinea pig kidney tissue was not precisely reproduced in the acetate extract. So experiments along the same line were tried with saline extract which was known to possess an inductive effect closely comparable to that of the original kidney tissue.

Minced tissue of guinea pig kidney (11.7 g.) was stirred in 18 cc. of 0.14 M NaCl solution for 1 hour at 5° C. and centrifuged for 30 minutes at 0° C. and 3,000 r.p.m. The same procedure was repeated on the centrifuged precipitate and the supernatants were combined. The pH of this saline extract was adjusted to 7.0. The ethanol-precipitate of this extract corresponds to "A Fraction" in the induction experiments by YAMADA and TAKATA (loc. cit.) and has been confirmed to induce spino-caudal structures at very high frequency. One aliquot of 8 cc. and one of 9 cc. of this saline extract were taken. To the former, 1 cc. of enzyme solution containing 2 mg. ribonuclease and 1 mg. desoxyribonuclease was added with 1 cc. of M/10 $MgCl_2$, and to the latter 1 cc. of M/10 $MgCl_2$ solution alone was added. These enzymes and $MgCl_2$ were dissolved in 0.14 M NaCl solution which was adjusted to pH 7.0 with N/10 NaOH. Incubation of the mixtures were conducted for 8 hours at 24° C. and pH 7.0 under shaking and dialyzing against 0.14 M NaCl solution of pH 7.0 containing $MgCl_2$. After incubation, an equal volume of ammonium sulphate solution, saturated at 5° C. and adjusted to pH 7.0 was added to each mixture. Keeping them for 30 minutes at 5° C., the precipitate was centrifuged down at 0° C. It was dissolved in cold 0.14 M NaCl solution of pH 6.8, and dialyzed in a cellophane bag against the same saline solution at 5° C. for 35 hours. During dialysis the mixtures were kept in constant motion on a shaker and the external media were renewed frequently. After making sure of the complete removal of ammonium sulphate, cold 95% ethanol was added to each dialyzed mixture and a protein fraction was obtained, as described in the preceding Experimental Groups.

The sample prepared after nuclease digestion was called Nase-A, and that without previous nuclease digestion was called con Nase-A. Nase-A was practically free of PNA and nucleotides (<0.1 PNA-P μg/mg N),[4] while con Nase-A showed PNA-P content of 21.8 μg/mg N.

Table III. The inductive ability of a protein fraction of saline extract of the kidney and the effect of exhaustive removal of PNA (Experimental Group III)

Experimental Series	Number of available explants	Number of explants with any induction	Number of explants containing induction types:													
			Somite	Notochord	Spinal cord	Deuterencephalon	Ear vesicle	Eye	Archencephalon	Nose	Lens	Pigment vesicle	Mesenchyme	Melanophore	Indifferent brain, or neural epithelium	Neural fragment
Nase-A	47	41	23	2	21	16	10	0	0	0	0	0	21	18	16	8
Con Nase-A	36	26	5	0	8	11	7	0	0	0	0	0	23	8	10	7

Nase-A: Implantation of PNA-free protein fraction of 0.14 M saline extract of guinea pig kidney, obtained by precipitation through one-half saturation of ammonium sulphate after nuclease treatment. *Con Nase-A*: Implantation of the same protein fraction without nuclease treatment and containing 21.8 PNA-P μg/mg N.

[4] Nase-A in Experimental Group III and RL-AC-1 and RL-AC-2 in Experimental Group IV showed no absorption whatever at 260 mμ either in their nucleic acid fraction by SCHNEIDER's method or in their extract with hot 5% trichloracetic acid. However, taking into consideration some possible experimental error and assuming the presence of the minimal quantity for detection, the possible maximal amount of PNA was calculated.

Results of Experimental Series Nase-A: By applying this PNA-free sample to the ectoderm explants, marked spino-caudal and deuterencephalic inductions were obtained; out of 47 explants, 41 contained inductions (Tables III and V). Among the latter, 20 could be classified into deuterencephalic, 29 into spino-caudal and one into unspecifiable types. No archencephalic induction was obtained (Figs. 9 and 10).

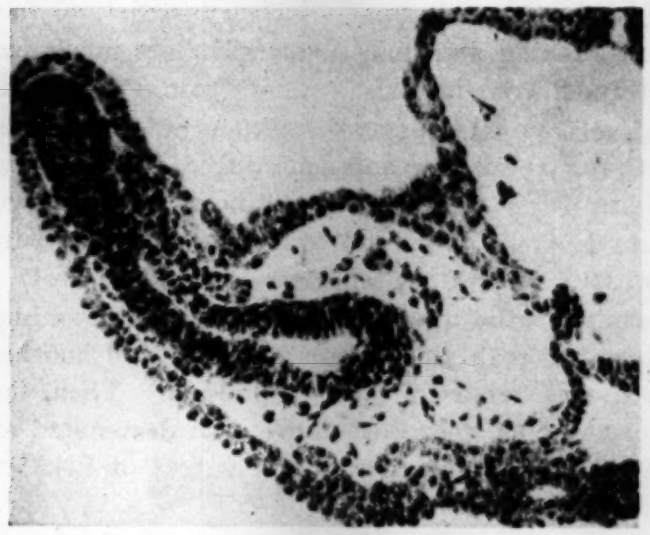

Fig. 9

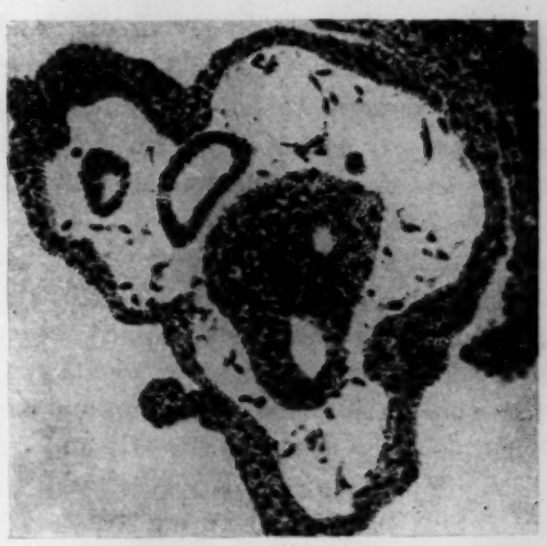

Fig. 10

Fig. 9. A spinal cord-type structure and mesenchyme induced by PNA-free protein fraction of saline extract of guinea pig kidney (Series Nase-A in Exp. Group III, ×75).

Fig. 10. Another section through the same explant. In the middle a deuterencephalon-like structure which continues to the spinal cord-type shown in the previous figure. Two ear vesicle-type structures and mesenchyme (× 75).

Results of Experimental Series Con Nase-A: Out of 36 explants, 26 showed induction; 14 were deuterencephalic, 9 spino-caudal character and 7 indifferent regional character (Fig. 11). A lower inductive frequency was observed in this control as compared with that of PNA-free Nase-A. However, this difference was found to be statistically insignificant. Hence, after complete removal of PNA the inductive frequency of the protein fraction of this saline extract was not diminished at all and its regional effect remained unchanged (Tables III and V.)

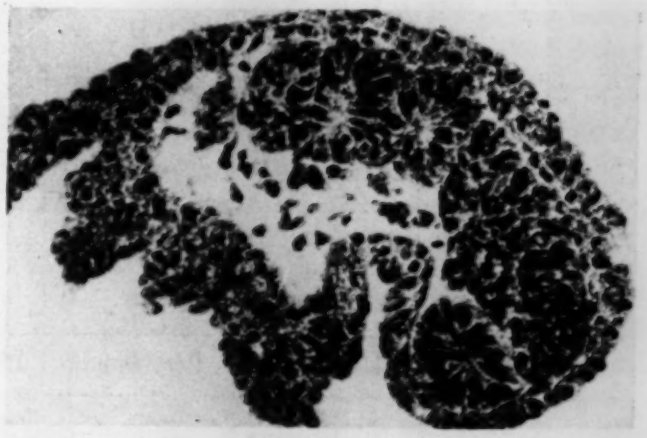

Fig. 11

Fig. 11. A series of somite-type structures with some mesenchyme induced by the control sample for Nase-A (Series con Nase-A in Exp. Group III, × 100).

E. Experimental Group IV.

Preparation of the samples, RL-PH, RL-AC-1, RL-AC-2:

Rat liver tissue weighing 4.2 g. was extracted with 15 cc. of phosphate buffer (N/10, pH

6.25). To an aliquot of the extract, cold 95% ethanol was added up to the final concentration of 75%. The precipitate was centrifuged down and served as the control for the samples with nuclease digestion. The precipitate was preserved in cold 95% ethanol and designated as RL-PH. This control sample contained 59.4 PNA-P μg/mg N.

Rat liver tissue of 1.3 g. was homogenized at 0° C. in 15 cc. of acetate buffer (N/10, pH 6.2) and extracted at 5° C. for 30 minutes. The pH of the extract was found to be 6.35. To 14 cc. of the acetate extract were added 2 cc. each of ribonuclease solution (1 mg/cc.), desoxyribonuclease solution (1 mg/cc.), and M/10 $MgSO_4$ solution. Nuclease digestion was continued for 7 hours at 20°-21° C. under constant shaking and dialyzing. Then the incubated mixture was brought to pH 6.8 by N/10 NaOH and to one-half saturation of ammonium sulphate at that pH and 5° C. by adding an equal volume of saturated solution of ammonium sulphate. The mixture was left standing at 5° C. for 30 minutes and centrifuged at 0° C. The precipitate was dialyzed against N/10 phosphate buffer of pH 6.2 for 38 hours at 5° C. and all ammonium sulphate was removed. The sample to be implanted was finally prepared by cold ethanol as described in other Experimental Groups and designated as RL-AC-1.

The supernatant which remained after precipitation by one-half saturation of ammonium sulphate was further brought to two-thirds saturation with ammonium sulphate and another fraction was precipitated. Ammonium sulphate was removed through dialysis. Then, the final sample was prepared by ethanol precipitation as described above, and designated as RL-AC-2. The samples, RL-AC-1 and RL-AC-2, were almost completely free of PNA or nucleotides ($<$ 0.5 PNA-P μg/mg N).[4)]

Table IV. The inductive ability of protein fractions of the liver and the effect of almost complete removal of PNA (Experimental Group IV).

Experimental Series	Number of available explants	Number of explants with any induction	Number of explants including induction types: Somite	Notochord	Spinal cord	Deuterencephalon	Ear vesicle	Eye	Archencephalon	Nose	Lens	Pigment vesicle	Mesenchyme	Melanophore	Indifferent brain, or neural epithelium	Neural fragment
RL-PH	32	28	0	0	0	0	0	7	17	12	5	4	6	11	4	13
RL-AC-1	41	27	0	0	0	0	0	4	15	5	5	2	5	7	2	17
RL-AC-2	35	34	0	0	0	0	0	15	30	12	11	3	16	4	3	17

RL-PH: Implantation of ethanol-precipitate of phosphate extract of rat liver, containing 59.4 PNA-P μg/mg N. *RL-AC-1:* Implantation of a PNA-free protein fraction of acetate extract of rat liver, precipitated by one-half saturation of ammonium sulphate after nuclease treatment. *RL-AC-2:* Implantation of a PNA-free protein fraction of acetate extract of rat liver, precipitated by two-thirds saturation of ammonium sulphate after nuclease treatment.

Results of Experimental Series RL-PH: As shown in Tables IV and V, in 28 out of 32 explants induction was observed, of which 22 showed archencephalic character, while 6 were regionally not specifiable (Fig. 12).

Results of Experimental Series RL-AC-1: Twenty-seven out of 41 explants

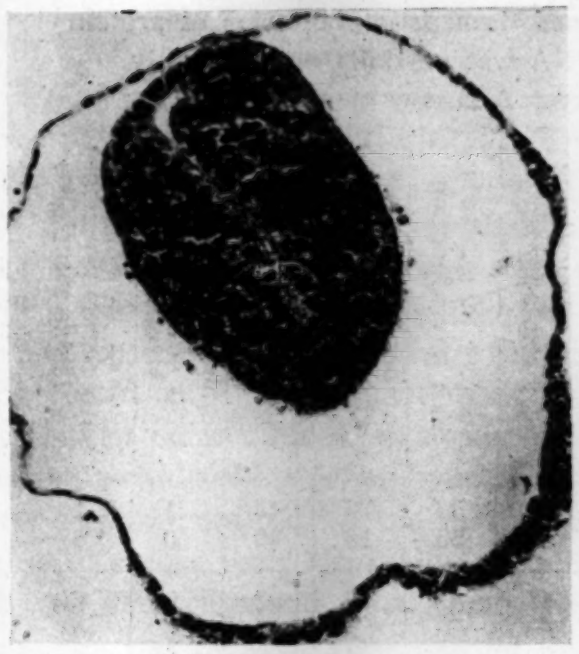

Fig. 12

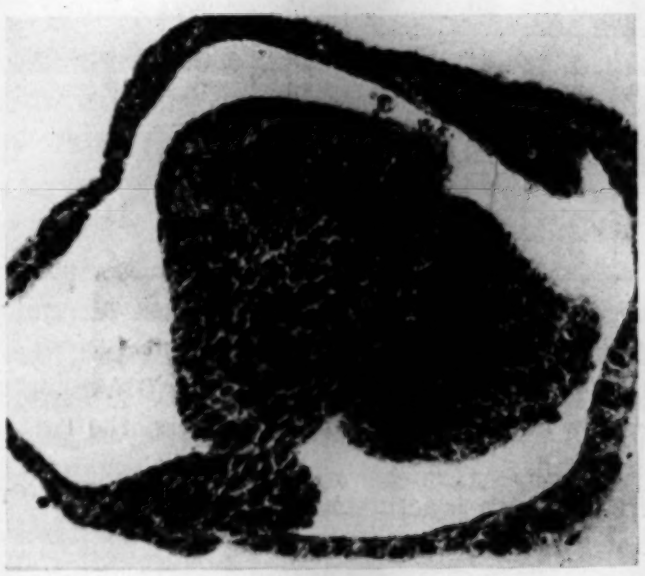

Fig. 14

Fig. 13

Fig. 12. An archencephalon-type structure induced by ethanol-precipitate of phosphate extract of rat liver (Series RL-PH in Exp. Group IV, × 95).

Fig. 13. A brain-like vesicle accompanied by two imperfect lentoids, induced by a PNA-free protein fraction of acetate extract of rat liver (Series RL-AC-1 in Exp. Group IV, × 70).

Fig. 14. Induction by another PNA-free protein fraction of the liver (RL-AC-2 in Exp. Group IV). Adjacent to the implant (left), an eye-like rudiment with accumulation of pigment. Two nose-type structures, one of which is shown with its opening. A large archencephalon-type structure continuous to the eye-like structure, is only suggested at the right of the section (× 85).

showed induction and 16 were of archencephalic character. In this series a slight decline of inductive frequency was observed (Fig. 13).

Results of Experimental Series RL-AC-2: Induction occurred in 34 out of 35 explants. Thirty-three explants showed archencephalic character (Fig. 14).

Thus the implants of this Experimental Group always induced archencephalic structures at high frequency irrespective of their PNA content (Tables IV and V). This means that the frequency and the regional character of induction were not changed by complete removal of PNA in the sample having archencephalic effect. It might be pointed out, however, that in the explants of Series RL-AC-1 a slight diminution of the size of the induced structures was generally observed. In addition, it must also be noted that PNA and nucleotides were almost completely removed from RL-AC-1 and RL-AC-2 while RL-PH contained a larger amount of PNA.

Table V. Regional effects of induction obtained through implantation of protein fraction of tissue extracts from which PNA was partially or completely removed through nuclease digestion. A survey of all series.

	Source of sample	Experimental Series	PNA content (PNA-P μg/mg N)	Number of available explants	Number of explants with any induction	Number of explants with spino-caudal structures	Number of explants with deuterencephalic structures	Number of explants with archencephalic structures	Number of explants with regionally non-specifiable structures
Guinea pig kidney	Phosphate extract	PH	9.0	54	54	26	44	1	1
		con PH+	21.1	52	50	18	40	0	6
	Acetate extract	AC++	7.4	47	43	5	32	5	8
		AC-1	0.9	50	50	5	44	3	4
		con AC-1+	7.9	32	30	4	25	2	4
		AC-2	0.7	46	41	1	33	10	3
		con AC-2++	6.2	39	38	8	32	2	6
	Saline extract	Nase-A*	<0.1	47	41	29	20	0	1
		con Nase-A+	21.8	36	26	9	14	0	7
Rat liver	Phosphate extract	RL-PH++	59.4	32	28	0	0	22	6
	Acetate extract	RL-AC-1*	<0.5	41	27	0	0	16	11
		RL-AC-2*	<0.5	35	34	0	0	33	1

* Nase-A, RL-AC-1 and RL-AC-2 showed no absorption at all at 260 mμ in their nucleic acid fraction after Schneider's method and in their extracts with hot 5% trichloracetic acid. Therefore, they were practically free of PNA, DNA and nucleotides. But taking into consideration possible experimental errors, the possible maximal amount of PNA was calculated.

\+ Control series testing the samples treated with inactivated enzymes.

++ Control series testing the samples not treated with enzymes.

Discussion

Brachet (1943) reported that in general the inductive ability of tobacco mosaic virus and particulate granules of the liver and of amphibian gastrula, among others, is proportional to their PNA content, and that when the devitalized organizer, the particulate granules of liver and the tobacco mosaic virus were treated with ribonuclease their inductive ability was strongly suppressed. He also reported that when the induction occurred, there could be observed a loss of PNA in the implant and simultaneous synthesis of PNA in the host ectoderm which was in contact with the implant. According to Brachet, corresponding changes in basophilia were also caused in the ectoderm explants which were subjected to sub-lethal cytolysis in a medium containing nucleic acids or nucleotides. These facts, together with his earlier observations concerning PNA distribution in early embryos, led him to assert that the inductive ability of devitalized tissues or their extracts is closely linked to PNA, and further that

PNA participates in the normal induction by the organizer (BRACHET, 1945, 1947, 1950).

However, recent experiments planned to examine that possibility revealed facts which are not favorable to his original idea. First, KUUSI (1951) could not show any clear correlation between PNA content and frequency or regionality of induction, when the inductive effects of guinea pig liver and its various fractions were tested. She also reported that no striking suppression of the inductive ability was observed when pieces of guinea pig kidney and liver digested with ribonuclease were inserted into the blastocoel of gastrula. In fact ,only a slight suppression of the archencephalic induction by liver was noticed, and no suppression of spino-caudal induction by kidney in spite of marked decrease in PNA content as judged by orcinol reaction. But the significance of these experiments is markedly impaired by the fact that control preparations incubated in distilled water also lost their PNA almost to the same or greater extent than those incubated with ribonuclease. Apparently she did not succeed in removing all PNA from the samples in view of the fact that there usually remains ribonuclease-resistant fraction of PNA after nuclease treatment (*c.f.* KUUSI, 1951. p. 53–55).

KUUSI (1953) further demonstrated that tobacco mosaic virus treated with 10% perchloric acid for 20 hours still induced archencephalic structures even after an almost complete removal of PNA. The frequency of induction caused by thus treated virus was 85% while that by untreated virus was 79%. She also reported that commercial PNA in albumin induced archencephalic structures at a high frequency, but that at the same time albumin alone caused archencephalic induction in the control series. Furthermore, she cultured explanted gastrula ectoderm in solutions containing commercial PNA or PNA extracted from rabbit liver. No neuralization was observed in *Triturus* ectoderm, while a small number of positive cases were obtained in *Amblystoma* ectoderm. Upon these experimental results, KUUSI could not draw a definite conclusion on the significance of PNA in the induction. When discussing the experimental results in her 1951 paper, KUUSI argued that in all probability the whole intact granules from the tissue but not their nucleic acid part might be responsible for the archencephalic induction. Further, she concluded from the above cited experiments with tobacco mosaic virus that the presence of PNA in the implant could enhance the archencephalic effect of the inducer, although PNA may not necessarily be involved in the archencephalic induction.

As one of the experiments with ribonuclease, data presented by ENGLÄNDER *et al.* (1953) may also be cited. They treated mouse kidney tissue with crystalline ribonuclease at 20°–40° C. for 2–6 hours. Nevertheless, the digested tissue induced archencephalic structures at the same frequency as in the control. Judged by the archencephalic structures induced, it appeared that the nuclease digestion might have strengthened the inductive effect of the tissue. They could not find regular changes in deuterencephalic and spino-caudal inductions by the ribonuclease-treatment. According to them, it might be depolymerized PNA or its derivatives rather than highly polymerized PNA which is essential for the archencephalic induction.

As included in KUUSI's later paper, BRACHET, KUUSI and GOTHIÉ (1952) con-

firmed that PNA removal was not accompanied by any drastic decrease in the inductive ability of tobacco mosaic virus, contrary to BRACHET's earlier experiments (1943), and they interpreted the discrepancy as being due to contamination of the ribonuclease used in the earlier experiments with proteolytic activity. They insisted, however, that at least in the process of normal induction pentose nucleoprotein is indispensable.

Doubts upon the role of PNA in induction were further advanced by OKAZAKI and OSAWA (1954). They reported that a protein fraction derived from the water extract of *Triturus*-gastrulae through treatment with ribonuclease had a powerful inductive ability when implanted in the gastrula blastocoel. The original water extract contained as much as 83.9% of PNA found in the *Triturus*-gastrulae, and the protein fraction derived from the extract treated with ribonuclease contained only 5% of PNA of the original water extract. However, the frequency of neural induction by PNA-rich and PNA-poor protein fractions showed no difference whatever. Moreover, LALLIER (1954) obtained inductions through implantation of devitalized organizer which had been stained with basic dyes, such as toluidine blue, acriflavin or acridine orange. He argued that the binding of these dyes with PNA would block the activity of PNA. The fact that neural induction was caused by the devitalized organizer with its PNA blocked in this way, should indicate that any role of PNA in the neural induction would be an indirect one.

Considering the data hitherto cited, it is highly probable that BRACHET's original idea is not applicable to all cases of induction. However, for a serious discussion of his idea, all these experiments seem to be inadequate because in many cases the removal of PNA was not checked quantitatively and in those cases checked, the removal was found to be incomplete. The experiments described in this paper clearly revealed that under the experimental conditions employed, an almost complete removal of PNA and its depolymerized derivatives or any marked reduction of PNA content did not change the inductive frequency to any significant extent. Here it might be also emphasized that spino-caudal and deuterencephalic induction (Series Nase-A in Experimental Group III) as well as archencephalic induction (Series RL-AC-1 and RL-AC-2 in Experimental Group IV) can be caused by implants from which PNA, desoxypentose nucleic acid, and nucleotides were almost completely removed.

On the other hand, participation of protein in the induction by adult tissues is suggested by the facts that spino-caudal and deuterencephalic effects of the saline extract of guinea pig kidney are almost completely abolished by a short treatment with trypsin and chymotrypsin (YAMADA, HAYASHI and TAKATA, 1954; YAMADA and TAKATA, in press).

Throughout the experimental series with acetate extract of guinea pig kidney (Series AC, AC-1, con AC-1, AC-2 and con AC-2), spino-caudal induction was less frequent while archencephalic induction more frequent when compared with other samples prepared from the kidney. As to the cause of this difference of regional effect of induction, the possibility is not excluded that pentose nucleoprotein was less extractable by acetate buffer than saline or phosphate buffer, and that the protein moiety of pentose nucleoprotein was responsible for the

spino-caudal induction.

Another possibility might be that the observed difference of regional inductive effect was caused by some difference in the state of proteins in acetate extract from those in saline or phosphate extracts. The latter possibility appears more probable in the light of the facts that spino-caudal and deuterencephalic effects of the ethanol-precipitate of 0.14 M saline extract of guinea pig kidney are changed into archencephalic effect by heat treatment or by bringing the extract to 3 M by adding sodium chloride (YAMADA and TAKATA, in press). A similar shift of regionality from spino-caudal towards archencephalic character is observed by treating a protein fraction of phosphate extract of guinea pig kidney with pepsin (YAMADA, HAYASHI and TAKATA, 1954; HAYASHI, unpublished).

These facts are easily explainable by the hypothesis proposed by YAMADA (1947, 1949, 1950 *a*, 1950 *b*), in which two factors, dorso-ventral and cephalo-caudal mediators, are assumed in the inducing system, controlling respectively the dorso-ventrality and cephalo-caudality of the differentiation of the reacting system. According to the hypothesis, both mediators should have the following morphogenetic effects: By the action of dorso-ventral mediator (M_{dv}) alone, the presumptive ectoderm is induced to form cephalic axial organs such as eye, fore-brain and nose (archencephalic induction). The co-operation of cephalo-caudal mediator (M_{cc}) with M_{dv} leads the presumptive ectoderm to differentiate caudal axial organs such as somite, notochord and spinal cord (spino-caudal induction). Deuterencephalic induction (induction of hind-brain and ear vesicle) is expected when M_{dv} operates together with a lower value of M_{cc} upon the presumptive ectoderm. Then, assuming that M_{cc} of tissue extracts is labile to heat, high salt concentration and pepsin treatmet, and M_{dv} is relatively stable, the selective inactivation of M_{cc} by these treatments inevitably results in a shift of inductive regionality from spino-caudal towards archencephalic character. The above cited experiments with trypsin and chymotrypsin, on the other hand, seem to indicate that M_{cc} and perhaps also M_{dv} can be inactivated by these proteolytic enzymes (YAMADA and TAKATA, in press).

In Experimental Group IV, where rat liver extract were used as inducers, a protein fraction treated with nucleases (RL-AC-1) showed a lower inductive frequency and smaller size of induced structures. On the other hand, the other protein fraction from the same experimental group treated with nucleases (RL-AC-2) showed a very high frequency of induction in spite of an almost complete removal of PNA and nucleotides. As the controls corresponding exactly to the Series RL-AC-1 and RL-AC-2 are lacking, the problem remains open whether the apparent reduction in the inductive frequency and the apparent diminution in the size of structures induced in Series RL-AC-1 are really significant. In this respect, further experiments are now in progress.

In parallel experiments, where solid samples of PNA were implanted in the ectoderm isolates, various salts of PNA failed to show any appreciable inductive ability when the isolates were cultured at pH 7.2. But in the isolates cultured at pH 7.7, sodium salt of PNA induced indifferent neural cells or archencephalic structures. These inductions were interpreted as due to sub-cytolytic stimulus

(YAMADA, TAKATA and OSAWA, 1954).

Taken altogether, the results presented in this paper seem to indicate that PNA is not the active inductive agent of the tissues here studied under our experimental conditions. This strengthens the idea that proteins participate in the mechanism of archencephalic, deuterencephalic and spino-caudal inductions caused by adult tissues under the present experimental conditions. As suggested earlier, experiments with proteolytic enzymes (YAMADA, HAYASHI and TAKATA, 1954) seems to support this idea. However, an immediate application of the present results to the normal organizer action might be dangerous. Some results obtained at our laboratory seem to suggest an important difference in the mechanism of the induction caused by the living organizer and that caused by the devitalized organizer or adult tissues.

SUMMARY

(1) Pentose nucleic acid (PNA) was removed from protein fractions of guinea pig kidney extracts and of rat liver extract by treating them with nucleases, and these treated protein fractions were tested for their inductive ability on the isolated ectoderm of early gastrula of *Triturus pyrrhogaster*.

(2) Deuterencephalic and spino-caudal structures can be induced by a protein fraction of phosphate extract of guinea pig kidney. The same protein fraction reduced in its PNA content by a nuclease digestion induced the same structures at a comparable frequency.

(3) When a protein fraction of acetate extract of guinea pig kidney was implanted, deuterencephalic induction was principally obtained. Almost indentical result was obtained by the same protein fraction reduced markedly in its PNA content.

(4) After an almost complete removal of PNA, a protein fraction of saline extract of guinea pig kidney showed no reduction at all in its inductive frequency and in its strong spino-caudal and deuterencephalic effects.

(5) When protein fractions of rat liver extract, from which PNA was almost completely removed, were tested, archencephalic structures were obtained at a high frequency.

(6) These results indicate that PNA is probably not the active inductive agent under the present experimental conditions. The suggestion has been made that under the experimental conditions employed, protein components might be involved in the process of archencephalic, deuterencephalic and spino-caudal inductions.

LITERATURE CITED

BRACHET, J., 1943. Pentosenucléoprotéides et induction neurale. Bull. Acad. Roy. Belg., Cl. Sci., Sér. *29*, 707–818. — BRACHET, J., 1945. Embryologie chimique. Liège, 2nd ed. — BRACHET, J., 1947. The metabolism of nucleic acids during embryonic development. Cold Spring Harbor Symp., *12*, 18-27. — BRACHET, J., 1950. Les caractéristiques biochimiques de la compètence et de l'induction. Rev. suisse Zool., *57*, 57–75. — BRACHET, J., T. KUUSI and S. GOTHIÉ, 1952. Une étude comparative du pouvoir en implantation et en micro-injection des acides nucléiques et des constituants cellulaires nucléoprotéiques. Arch. Biol., *63*, 429–440. — CERIOTTI, G., 1952.

A microdetermination of desoxyribonucleic acid. J. Biol. Chem., *198*, 297–303. — CHUANG, H. H., 1938. Spezifische Induktionsleistungen von Leber und Niere im Implantatversuch. Biol. Zentralbl., *58*, 472–480. — CHUANG, H. H., 1939. Induktionsleistungen von frischen und gekochten Organteilen (Nieren, Leber) nach ihrer Verpflanzung im Explantate und verschiedene Wirtsregionen von *Triton*-keimen. ROUX' Arch., *139*, 556–638. — CHUANG, H. H., 1940. Weitere Versuche über die Veränderung der Induktionsleistungen von gekochten Organteilen. ROUX' Arch., *140*, 25–38. — ENGLÄNDER, H., A. G. JOHNEN and W. VAHS, 1953. Untersuchungen zur Klärung der Leistungsspezifität verschiedener abnormen Induktoren bei der Embryonalentwicklung der Urodelen. Experientia, *9*, 100–101. — FUJII, T., 1941. Mesoderm induction obtained with frog white skin, and neural induction obtained with pyrrol-chrome and black skin. J. Fac. Sci. Tokyo Imp. Univ. sec. IV, *5*, 425–432. — FUJII, T., 1944. Experimental studies on neural and mesodermal inductions in the early development of *Triturus*. J. Fac. Sci. Tokyo Imp. Univ. sec. IV, *6*, 451–475. — HAMA, T., 1944. On the inductive specificity of fresh and boiled tissues of vertebrate kidney and liver. Annot. Zool. Jap., *22*, 165–172. — HOLTFRETER, J., 1934. Über die Verbreitung induzierender Substanzen und ihre Leistungen im Triton-Keim. ROUX' Arch., *132*, 308–383. — KAWAKAMI, I. and I. KAWAKAMI, 1947. On the inductive effect of lens of adult animals (in Japan.). Zool. Magaz. (Tokyo), *57*, 81–82. — KUUSI, T., 1951. Über die chemische Natur der Induktionsstoffe mit besonderer Berücksichtigung der Rolle der Proteine und der Nukleinsäure. Ann. Zool. Soc. "Vanamo", *14*, 1–98. — KUUSI, T., 1953. Sur les effets des acides nucléiques et des protéines dans l'induction héterogène. Arch. Biol., *64*, 189–226. — LALLIER, R., 1954. Effet de quelques colorants basiques sur l'induction embryonnaire. Experientia, *10*, 222. — LEVY, M. and A. H. PALMER, 1940. Iodometric estimation of small quantity of nitrogen without distillation. J. Biol. Chem., *136*, 57-60. — LORING, H. S., F. H. CARPENTER and P. M. ROLL, 1947. The hydrolysis of yeast ribonucleic acid by ribonucleinase. I. The extent of hydrolysis and the preparation of ribonucleinase-resistant fraction after ribonucleinase treatment. J. Biol. Chem., *169*, 601–608. — OKADA, YÔ K., 1948. Induction of notochord by frog ventral skin and the organizer effect of such a notochord. Proc. Japan Academy, *24*, 22–29. — OKAZAKI, R. and S. OSAWA, 1954. Inductive effect of protein fractions obtained from *Triturus*-gastrulae (in Japan.). Zool. Magaz. (Tokyo), *63*, 174–175. — ROTMANN, E., 1942. Zur Frage der Leistungsspezifität abnormer Induktoren. Naturwiss., *30*, 60-62. — SCHNEIDER, W. C., 1945. Phosphorus compounds in animal tissues. I. Extraction and estimation of desoxypentose nucleic acid and of pentose nucleic acid. J. Biol. Chem., *161*, 293-303. — TOIVONEN, S., 1940. Über die Leistungsspezifität der abnormen Induktoren im Implantatversuch bei *Triton*. Ann. Acad. Sci. Fenn. Ser. A, *55*, 1–150. — TOIVONEN, S., 1945. Zur Frage der Induktion selbständiger Linsen durch abnorme Induktor in Implantatversuch bei *Triton*. Ann. Zool. Soc. "Vanamo", *11*, 1–28. — TOIVONEN, S., 1949. Die Induktionswirkung von leistungsspezifischen heterogenen Induktoren nach Behandlung mit verschiedenen Eingriffen (Temperatur, Lösungsmitteln u. dgl. m.) im Implantationsversch bei *Triton*. Arch. Soc. Zool. "Vanamo", *4*, 28-34. — TOIVONEN, S., 1950. Stoffliche Induktoren. Rev. suisse Zool., *57*, 41–56. — TOIVONEN, S., 1953. Bone-marrow of the guinea pig as a mesoderm inductor in implantation experiments with embryos of *Triturus*. J. Embryol. exp. Morph., *1*, 97–104. — TOIVONEN, S., and T. KUUSI, 1948. Implantationsversuche mit in verschiedener Weise vorbehandelten abnormen Induktoren bei *Triton*. Ann. Zool. Soc. "Vanamo", *13*, 1–19. — YAMADA, T., 1947. An extension of the potential theory of the morphogenesis (in Japan.). Zool. Magaz. (Tokyo), *57*, 124–126. — YAMADA, T., 1949. Double-potential theory of the early ontogenesis (in Japan.). "New Biology", 85–116. — YAMADA, T., 1950 *a*. Dorsalization of the ventral marginal zone of the *Triturus* gastrula. I. Ammonia-treatment of the medio-ventral marginal zone. Biol. Bull., *98*, 98–121. — YAMADA, T., 1950 *b*. Regional differentiation of the isolated ectoderm of the *Triturus* gastrula induced through a protein extract. Embryologia, *1*, 1–20. — YAMADA, T., 1952. Nucleoprotein and regional induction (in Japan.). Zool. Magaz. (Tokyo), *61*, 115. — YAMADA, T. and K. TAKATA, 1955. An analysis of spino-caudal induction by the guinea

pig kidney in the isolated ectoderm of the *Triturus*-gastrula. J. Exp. Zool., *128*, No. 3 (in press). — YAMADA, T. and K. TAKATA, Effect of trypsin and chymotrypsin on the inducing ability of the kidney and its fractions. Exp. Cell Research, Suppl. (in press). — YAMADA, T., Y. HAYASHI and K. TAKATA, 1954. Enzymatic analyses of regional inductive effect of tissue extracts (in Japan.). Zool. Magaz. (Tokyo), *63*, 451. — YAMADA, T., K. TAKATA and S. OSAWA, 1954. A test of the inductive effect of pentose nucleic acid of the kidney on the isolated ectoderm. Embryologia, *2*, 123–131. — ZITTLE, C. A., 1946. Ribonucleinase. IV. Hydrolysis of ribonucleic acid by ribonucleinase and by sodium hydroxide. J. Biol. Chem., *163*, 119–127.

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